The Role of Dopamine and Glutamate in Associative Learning by the Pigeon (*Columba livia*)

Martín Javier Acerbo

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Die Rolle von Dopamin und Glutamat beim

Assoziativen Lernen der Taube (*Columba livia*)

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Martín Javier Acerbo

Referent 1: Prof. Dr. Juan D. Delius, Universität Konstanz
Referent 2: Prof. Dr. Michael Koch, Universität Bremen
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Martín Javier Acerbo

Referee 1: Prof. Dr. Juan D. Delius, University of Konstanz
Referee 2: Prof. Dr. Michael Koch, University of Bremen
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Contents

Zusammenfassung

Assoziatives Lernen beinhaltet das Erlernen einer Verbindung zwischen Repräsentationen zweier Ereignisse (zwei Reize oder ein Reiz und eine Reaktion). Danach kann das Auftreten eines dieser Ereignisse die Repräsentation des anderen aktivieren. Um die Beteiligung des glutamatergen und dopaminergen Systems beim Lernen zu untersuchen, wurden zwei assoziative Lernparadigmen verwendet. Apomorphin-induziertes und ein simultanes visuelles Diskriminationlernen wurden benutzt, um die Beteiligung der beiden zuvor genannten Übertragungsmechanismen zu untersuchen.

Kapitel I gibt eine kurze Einführung in das Thema "Lernen". Insbesondere das klassische Konditionieren, sowie die Bedeutung von Drogen und kontextuellen Reizen bei der klassischen Konditionierung werden vorgestellt. Ferner ihre Bedeutung beim Diskriminationslernen, sowie für Farbpräferenzen bei Vogelarten und Tieren im allgemeinen. Diese kurze Einführung in das dopaminerge und glutamaterge System dient dazu, ein assoziatives Lernmodell (Wickens, 1990) einzuführen. Dieses Modell soll als Hintergrund für das Design und die Interpretation der im folgenden beschriebenen Experimente dienen.

Im Kapitel II werden das glutamaterge und dopaminerge System bei Wirbeltieren besprochen. Neben einer detaillierten Beschreibung des glutamatergen Systems wird auch an dieser Stelle seine Relevanz im Wirbeltiergehirn analysiert. Die Molekülsynthese bis hin zum wichtigsten glutamatergen Prozess beim Lernen, der sogenannten Langzeitpotenzierung (LTP), werden vorgestellt. Rezeptoren werden beschrieben und ihre Funktion in den unterschiedlichen glutamatergen Bahnen besprochen. Besondere Aufmerksamkeit wurde dem NMDA-Rezeptor gewidmet, der ein ionotropischer Kanalrezeptor ist. Die glutamaterge, cortiko-striatale Bahn ist von besonderer Bedeutung für das Lernen. Folglich wird sie ebenfalls zusammen mit den übrigen dazugehörigen Leitungsbahnen vorgestellt. Die Wirkung von Dopamin auf das Verhalten von Tieren, ihre Synapsen sowie der Metabolismus der dopaminergen Neurotransmitter, werden beschrieben. Dopaminerge Gehirnfunktionen, sowie die dopaminergen Rezeptoren im Kontext des Verhalten von Tieren werden ebenso behandelt, wie die Wirkungsweise von agonistischen und antagonistischen Dopaminneurotransmitter auf eben dieselbe Verhaltensweisen.

Das dopaminerge System des Wirbeltier Taube Gehirns wurde bislang relativ gut erforscht. Nichtsdestotrotz gibt es zur Zeit bezüglich des Rückenmarks noch einige offene Fragen. Da es einige widersprüchliche Befunde hinsichtlich der Zytoarchitektur des dopaminergen Systems im Rückenmark adulter Vögel gibt, wurde das dopaminerge System

bei Vögeln nicht erschöpfend untersucht. Einige Studien wurden mit embryonalen oder jungen Küken oder japanischen Wachteln ausgeführt. Die Mehrzahl der anderen Studien konzentrierten sich jedoch auf Säuger, Reptilien, sowie knorpelartige Fische. Bezüglich dieses Systems bei erwachsenen Vögeln besteht somit eine Wissenslücke. Um immunozytochemische Markierungen von DA-, TH- und DARPP-32-immunoreaktiven Zellen und Axonen durchführen zu können, wurden Antisera gegen Dopamin (DA), Tyrosin-Hydroxylase (TH, Enzym der dopaminergen Biosynthese) und DARPP-32 (ein Phosphoprotein, das mit D1-ähnlichen Dopaminrezeptoren gekoppelt ist), verwendet. Der Befund, dass 2 DA-immunoreaktive Zellpopulationen mit den TH-immunoreaktiven Zellen kolokalisiert waren, wird in Bezug auf die Phylogenese des dopaminergen Systems bei Wirbeltieren und knorpelartige Fischen diskutiert.

Kapitel III präsentiert eine differenzierte Erläuterung der Apomorphin-Kontext-Konditionierung. Dieses Paradigma wurde verwendet, um die dopaminerge und glutamaterge Beteiligung am assoziativen Lernen zu untersuchen. Apomorphin ist ein dopaminerger Rezeptoragonist. Wiederholte Injektionen dieses Neurotransmitters rufen bei Tauben stereotype Pickanfälle hervor. Diese Pickreaktion steigert sich graduell (bzw. sensitisiert) bis zu einer dosisabhängigen Asymptote an. Solch apomorphin-induziertes Picken kann auf zwei Arten erklärt werden. Die sogenannte “pharmakologische Hypothese” postuliert, dass die Picksensitisierung eine Konsequenz pharmakologischer oder biochemischer Änderungen ist, die von Apomorphin angestossen werden und direkt die synaptische Effizienz ändern. Auf der anderen Seite geht die “Konditionierungshypothese” davon aus, dass Apomorphine einen Lernprozess initiiert, der wiederum die synaptische Effizienz verändert. Das bedeutet, dass wiederholte Gabe von Apomorphin im selben experimentellen Kontext in einer Pawlowschen Konditionierung resultieren kann. Die Apomorphinwirkung kann dann als unkonditionierter Reiz (US) angesehen werden und die kontextuellen Reize als konditionierte Stimuli (CS). Eine Sensitisierung wäre dann das Ergebnis des spontanen Pickens (der unkonditionierten Reaktion, UR), das direkt von Apomorpin induziert wird, zuzüglich der Entwicklung einer konditionierten Reaktion (CR), die vom Kontext hervorgerufen wird. Hierzu wird eine Standardkurve der Apomorphinsensitisierung vorgestellt. Lindenblatt und Delius (1987) zeigten, dass wenn Apomorphin im selben experimentellen Käfig verabreicht wird, die Picksensitisierung auf die visuelle Kontextreize klassisch konditioniert werden kann. Um das Langzeitgedächtnis von Tauben bezüglich einer erworbenen Apomorphin-Kontext-Assoziation zu prüfen, wurde ein Experiment zum apomorphin-induzierten Lernen durchgeführt und im diesem Kapitel vorgestellt. Dazu wurden Tauben zwei Jahre nach dem

Erwerb der Assoziation unter denselben Bedingungen (Dosis, Kontext) getestet. Es konnte gezeigt werden, dass die Tiere die Apomorphin-Kontext-Assoziation immer noch erinnerten. Dieser Befund unterstützt die Konditionierungshypothese der Picksensitisierung: Zwei Jahre stellen eine ausreichende Zeitspanne dar, um jegliche pharmakologischen Effekte, die Apomorphin direkt auf die dopaminergen Rezeptoren gehabt haben könnte, rückgängig zu machen. Nur synaptische Veränderungen, die durch einen Lernprozess bewirkt worden, dürften eine solche Zeitspanne überdauern können.

Gegenstand von Kapitel IV sind die möglichen Änderungen an dopaminergen Synapsen infolge von Apomorphingaben. Anzahl und Qualität von Dopaminrezeptoren wurden bei Tauben gemessen, die in ihren Heimkäfigen chronisch mit Apomorphin behandelt wurden. Die Messergebnisse wurden mit denen von Tauben verglichen, die unter denselben Bedingungen nur Salineinjektionen erhielten. Die Rezeptoren wurden mittels einer Binding-Assay-Technik ermittelt. Dabei wurden zwei radioaktiv markierte dopaminerge Antagonisten verwendet: der Antagonist [^{3}H]-SCH-23390, der spezifisch für Rezeptoren des D1-Typs ist, sowie der Antagonist [^{3}H]-Spiperone, der spezifisch für Rezeptoren des D2-Typs ist. Tauben, die chronisch mit Apomorphin behandelt wurden, wiesen gegenüber Kontrolltieren eine erhöhte Anzahl an D1-Rezeptoren auf, sowie eine erniedrigte Anzahl an D2-Rezeptoren. Die Ergebnisse dieses Experimentes legen die Vermutung nahe, dass dopaminerge Rezeptoren infolge von dauerhafter Apomorphinverabreichung verändert werden. Aufgrund dieser Ergebnisse kann jedoch nicht entschieden werden, ob diese Veränderungen die pharmakologische oder die Konditionierungshypothese unterstützen. Der Grund hierfür ist, dass in der genannten Studie die Tauben den Heimkäfig mit den Apomorphineffekten assoziiert hätten können. Beim Experiment Nummer 4 handelt es sich um eine Vorstudie, die ermitteln sollte, in welcher Gehirnregion Apomorphin wirken könnte, wenn es die stereotype Reaktion des Pickens induziert. In zwei Hirnregionen wurden Kanülen direkt implantiert: in den Nucleus accumbens septi, sowie in eine kaudale Region des Striatums, die das Paleostriatum primitivum und Paleostriatum augmentatum umfasst. Sobald die Kanülen bilateral (eine in jeder Hirnhälfte) in den genannten Hirnregionen implantiert waren, wurde Apomorphin verarbreicht: zunächst intramuskulär, um die Tauben zu sensitisieren und später intrakranial, um zu testen, ob Injektionen wirksam waren in einer der bereits erwähnten Hirnregionen die Pickreaktion aufrechterhalten würden. Die Ergebnisse dieses Experimentes legen nahe, dass der Nucleaus accumbens, nicht jedoch die kaudale Region des Striatums an apomorphin-induzierten Picken beteiligt ist. Dieses Ergebnis wird später zusammen mit den Experimenten aus Kapitel 7 ausführlicher diskutiert werden.

Ein zweites assoziatives Lernparadigma wird in Kapitel V entwickelt. Es beinhaltet eine simultane visuelle Diskriminationsaufgabe, wobei 6 verschiedene Farben gleichzeitig angeboten werden, jedoch nur eine auf Futter hinweist. Diese Diskriminationsaufgabe wurde mit dem Ziel entwickelt, einen schnellen und konsistenten Lernprozess untersuchen zu können. Die Aufgabe stellt eine Weiterentwicklung der Wahl-nach-Muster-Experimente von Wright und Delius (1994) mit Kiesreizen dar. Sechs Näpfe mit unterschiedlich farbigem Kies werden simultan dargeboten. Nur in einem Napf bedeckt der Kies das Futter, d.h. nur eine Farbe ist mit Futter assoziiert und damit ein positiver Reiz (S+). Die verbleibenden 5 Kiessorten/Farben stellen negative Reize dar (S-). Um Futter zu finden, müssen die Tauben im Kies suchen. Während des Tests enthalten die Näpfe zwar dieselben Kiessorten wie im Training, jedoch kein Futter. Sowohl Wahlen als auch Ausmass der Pickreaktionen auf den S+ wurden aufgezeichnet. Experiment 5 bestand aus 2 Phasen. In der ersten wurden zwei Gruppen von Tauben trainiert, entweder einen von 6 farbigen Kiesarten zu diskriminieren, oder nicht zwischen den Kiesarten zu diskriminieren (in jedem Napf wurde Futter versteckt). Die Ergebnisse dieser ersten Phase zeigten, dass die Tauben sehr schnell eine Kiesfarbe-Futterassoziation bildeten. Im Test zeigten sie eine hohe Präferenz für den S+. Die Tiere der nicht-diskriminativen Gruppe zeigten hingegen-abgesehen von einigen spontanen Farbpräferenzen-keine spezielle Präferenz. Anschliessend wurden beide Taubengruppen einer zweiten Prozedur unterzogen. Sie wurden trainiert, einen zweiten, vom ersten S+ verschiedenen S2+ zu lernen. Hiermit sollte ermittelt werden, in welchem Ausmass ein Diskriminations- und ein nicht-diskriminatives Training ein nachfolgendes Erlernen eines anderen positiven Reizes beeinflusst. Tauben, die zuvor ein nicht-diskriminatives Training durchliefen, fiel es schwerer, eine Assoziation mit einem neuen Reiz zu erwerben (diese Tauben führten zum ersten Mal eine Diskrimiantionsaufgabe), verglichen mit Tauben, die zuvor einen anderen positiven Reiz diskriminieren mussten. Diese Ergebnisse werden in Bezug auf Wiederlernen und latente Hemmungsprozesse diskutiert.

Im folgenden wurde diese Kies-Futter-Assoziation verwendet, um die Beteiligung der dopaminergen und glutamatergen Übertragungsmechanismen beim Assoziations- und Diskriminationslernen zu untersuchen. Im Rahmen des assoziativen Paradigmas wurden in Kapitel VI drei verschiedene Antagonisten getestet. Der D2-Rezeptor-Antagonist Haloperidol wurde im Experiment 6 verwendet, um die Hypothese zu untersuchen, ob D2-Dopamin-Rezeptoren eine Rolle für das Lernen der assoziativen und diskriminativen Aufgabe spielen. Haloperidol wurde in zwei unterschiedlichen Taubengruppen und zwei verschiedene Dosierungen während des Trainings verabreicht. Eine dritte Gruppe erhielt nur Saline. Das

Training bestand darin, einen vorgegebenen S1+ zu diskriminieren. Alle Gruppen erhielten während des Tests Salineinjektionen. Während der Erwerbsphase wurden keinerlei Beeinträchtigungen beobachtet. Dies erlaubt die Vermutung, dass der Erwerb dieser Diskriminationsaufgabe nicht durch D2-Rezeptoren vermittelt wird. Mit einem vergleichbaren Versuchsplan wurde die Rolle der D1-Rezeptoren beim Abruf des Gelernten untersucht. Eine zusätzliche Gruppe wurde verwendet. Die zugehörigen Tauben wurden mit Saline injiziert. Saline wurde während des Trainings appliziert, während im Test SCH-23390 benutzt wurde. Sowohl Antagonisten als auch Salinelösung wurden intrakranial verabreicht (Paleostriatum augmentatum und Paleostriatum primitivum). Verglichen mit der erwarteten Reaktion für die anderen Tiere, welche die Aufgabe ohne Schwierigkeiten lösten, zeigten die mit Saline behandelten Tauben eine überraschend niedrigere Reaktion. Aufgrund eben dieses Umstands, hatten die Tauben unter dem D1-Rezeptorantagonisten SCH-23390 höhere Reaktionsniveaus, sodass die folgenden Ergebnisse mit Vorsicht interpretiert werden sollten. Beim Erwerb und Abruf ergab sich in dieser Diskriminationsaufgabe kein Blockierungseffekt. Das legt nahe, dass D1-Rezeptoren bei dieser Aufgabe nicht beteiligt sind. Experiment 8 untersuchte die Bedeutung von NMDA für diese Diskriminationsaufgabe. Dasselbe Design wie im vorausgehenden Versuch wurde verwendet. Als NMDA Rezeptorantagonist diente MK-801. NMDA-Rezeptoren spielten keine Rolle für Erwerb und Abruf bei dieser Diskriminationsaufgabe. Aufgrund der Ergebnisse mit der Assoziations- und Diskriminationsaufgabe, die hier verwendet wurden, muss angenommen werden, dass es nicht einen einzigen molekular-assoziativen Mechanismus gibt, der den Ergebnissen aller Kernexperimente zugrundeliegt.

Das andere assoziative Lernparadigma, das um die Beteiligung des dopaminergen und glutamatergen Systems zu untersuchen in dieser Arbeit verwendet wurde, ist das apomorphin-induzierte Lernen. Innerhalb dieses Paradigmas wurden dieselben Drogen verwendet, die zuvor für die Diskriminationsaufgaben verwendet wurden. Obwohl das Apomorphin-Kontext-Paradigma in unserem Labor häufig verwendet wurde, gab es bislang nur ein Experiment, in dem die Beteiligung von dopaminergen D2-Rezeptoren untersucht wurde. Hierbei untersuchte Godoy (2000) den Einfluss von Haloperidol auf die Apomorphin-Kontext-Konditionierung. In Kapitel VII wurden die folgenden Experimente auf der Basis dieses Experimentes entwickelt und ausgeführt. Während des Trainings hatten die Tauben entweder nur Haloperidol, nur Apomorphin oder Haloperidol zusammen mit Apomorphin bekommen. Während des Tests erhielten alle Gruppen Saline (Godoy hatte in einem Test den Tieren Apomorphin verabreicht). Die Ergebnisse dieses Experimentes lassen vermuten, dass

Haloperidol den Erwerb des apomorphin-induzierten Lernens nachhaltig beeinträchtigt. Dies bedeutet, dass D2-Rezeptoren für den Erwerb dieses Assoziationslernens wichtig sind, nicht jedoch für den Abrufprozess (letzteres hatte Godoy's Versuch gezeigt). Die Rolle der D1-Rezeptoren wurde in Experiment 10 untersucht. Um den Effekt von SCH-23390 auf den Abruf zu untersuchen, wurde ein vergleichbares Design wie beim Experiment 9 verwendet, jedoch mit der Einbeziehung einer zusätzlichen Gruppe. Die Ergebnisse dieses Experimentes stützten die Hypothese, dass D1-Rezeptoren beim Erwerb, aber nicht beim Abruf von assoziativem Lernen involviert sind. Diese beiden letzten Experimente demonstrieren also, dass sowohl D1- als auch D2-Rezeptoren für den Erwerb, nicht jedoch für den Abruf dieses Assozationslernens wichtig sind. Ein ähnliches Design wurde verwendet, um die Beteiligung von NMDA an diesem Assoziationslernen zu untersuchen. Experiment 11, in dem der NMDA Rezeptorantagonist MK-801 eingesetzt wurde, legt nahe, dass NMDA-Rezeptoren sowohl für den Erwerb als auch für den Abruf wichtig sind. Diese Ergebnisse werden auf dem Hintergrund des assoziativen Lernmodells von Wickens (1990) diskutiert.

In letztem Kapitel dieser Arbeit wird eine allgemeine Diskussion aller experimentellen Befunde vorgestellt. Besondere Aufmerksamkeit wird hier auf das assoziative Lernen gewidmet und auf die Frage, inwieweit die Ergebnisse zum Modell von Wickens passen. Die wesentliche Schlussfolgerung ist die, dass das assoziaitive Lernen ein eher vielseitiger Prozess ist, in dem zahlreiche verschiedene Mechanismen involviert sein könnten. Weder das assoziative noch das diskriminative Paradigma können mit dem oben genannten Modell in Einklang gebracht werden, während das Apomorphin-induzierte Lernen eine gute Übereinstimmung zeigt. Beide Paradigmen weisen einige Gemeinsamkeiten auf, z.B. Futteraufnahmeverhalten und visuelle Erkennung der Reize. Es konnte gezeigt werden, dass das Apomorphin-induzierte Picken neuronal anders aktiviert wird, als hunger-induziertes Picken. Andererseits scheinen assoziative Mechanismen im Striatum zwischen glutamatergen und dopaminergen Systemen, zumindestens für einige Arten des assoziativen Lernens, funktional zu sein. In jedem Falle sollte die glutamaterge und dopaminerge Beteiligung beim assoziativen Lernen in weiteren und neuen Verhaltensparadigmen untersucht werden.

Abstract

Associative learning involves learning a connection between two events (two stimuli or a stimulus and a response) after their presentation so that the posterior occurrence of one of them activates the representation of the other. In the present dissertation, two associative learning paradigms are used in order to study the role of the glutamatergic and dopaminergic systems on learning. Apomorphine-induced learning and a simultaneous visual discrimination task are used to assess the participation of both systems mentioned above.

Chapter I includes a brief introduction to learning (especially classical conditioning), the use of drugs and of contextual cues as stimuli in this domain. It also treats discrimination learning and colour preferences in birds and in animals in general. The two paradigms used in this work are briefly described in the context of dopamine and glutamate involvement. A brief introduction to dopaminergic and glutamatergic systems introduces the associative learning model proposed by Wickens (1990). This model will be used as a basis for the design and interpretation of the following experiments.

Both the glutamatergic and dopaminergic systems in vertebrates are discussed in chapter II. A detailed description of the glutamatergic system is given and its relevance for the brain of vertebrates is analysed. Glutamate molecule synthesis and the most relevant glutamatergic process for learning, the long-term potentiation (LTP), are described. Further, a detailed description of the glutamatergic receptors as well as a general view of their participation in the different glutamatergic pathways are revised. Special emphasis falls on the NMDA glutamate receptor (an ionotropic channel receptor). Because of its critical role in learning, the corticostriatal glutamatergic pathway together with the remaining glutamatergic pathways is described. The hypothetical involvement of LTP in learning is discussed. Also a description of dopamine action mechanisms on animal behaviour is given. Dopaminergic synapses, as well as the metabolism of dopamine are included here. The dopaminergic brain functions, dopaminergic receptors (in the context of their actions on animal behaviour), the action mechanisms of dopaminergic agonists and antagonists on this behaviour are described. The dopaminergic system at a telencephalic level is very well understood. Nevertheless, at the level of the spinal cord, some aspects remain inconclusive. The dopaminergic system in birds has not been exhaustively studied because of the unclear data regarding its cytoarchitecture in the spinal cord of adult birds. Some studies have been done in embryo and hatching chicks and in Japanese quails. Many others were performed in mammals, reptiles, osseous and cartilaginous fish. However, in adult birds, a lack of knowledge about this system persists.

Antisera against dopamine (DA), tyrosine hydroxylase (TH, an enzyme of the dopamine biosynthetic pathway) and DARPP-32 (a phosphoproteine linked to the D1-like dopamine receptor) were used to perform immunocytochemistry labelling of the DA-, TH- and DARPP-32-immunoreactive cells and fibres. The finding of two DA-immunoreactive cells populations co-localised by means of the TH-immunoreactive cells are discussed in the light of the phylogenetic evolution of the dopaminergic system in both vertebrates and cartilaginous fish.

Chapter III provides a detailed explanation of apomorphine-context conditioning. This paradigm is then used to test the dopaminergic and glutamatergic roles in associative learning. Apomorphine is an dopaminergic receptor agonist. Repeatedly administrated to pigeons, it induces bouts of a stereotyped pecking response. This pecking response gradually increases or sensitises up to a dose-dependent asymptotic value. Apomorphine-induced pecking can be explained by two alternative hypotheses. On the one hand, the “pharmacological hypothesis” sees the pecking sensitisation as the result of the pharmacological or biochemical changes induced by apomorphine, which directly modify the efficacy of the synapses. On the other hand, the “conditioning hypothesis” postulates that apomorphine initiates the learning process, which in turn modifies the efficacy of the synapses. This means that repeated apomorphine administration in the same experimental context results in a Pavlovian association: the apomorphine effects act as an unconditioned stimulus (US) and the contextual cues act as a conditioned stimulus (CS). Accordingly, the sensitisation would then be the result of the spontaneous pecking (directly induced by apomorphine), the unconditioned response (UR) and the developing conditioned pecking response (CR) elicited by the context. A standard curve of apomorphine sensitisation and saline control is shown. When apomorphine is administrated in the same experimental cage, pecking sensitisation becomes classically conditioned to visual contextual cues, as demonstrated by Lindenblatt & Delius (1987). In addition, a first experiment using apomorphine-induced learning is presented here. The aim of this experiment is to test the memory of pigeons previously trained in apomorphine-context association. Pigeons who underwent the apomorphine paradigm were selected and tested in the same condition as two years before. Both apomorphine doses and contextual cage were the same. It is demonstrated that pigeons are capable of remembering the apomorphine-context association after this two-year interval. Since two years can be considered to be long enough to negate any pharmacological effect of apomorphine acting directly on the dopaminergic receptors, this finding supports the conditional hypothesis of pecking sensitisation: only a synaptic modification due to learning processes seems to be strong enough to survive such a prolonged time period.

Possible changes in the dopaminergic synapses due to apomorphine administration are discussed in chapter IV. The amount and quality of dopamine receptors in pigeons that received chronical apomorphine treatment in the home cage and in those receiving only saline solution injections was measured. This was done by means of a binding assay technique which utilised [^{3}H]-SCH-23390 (a radiolabelled dopaminergic antagonist specific for D1-like receptors) and [^{3}H]-Spiperone (a radiolabelled specific D2-like receptor antagonist). An increase in the amount of D1-like receptors and a decrease in the number of D2-like receptors in pigeons that received a chronically apomorphine treatment was observed. This becomes clear when comparing the relative number of receptors (D1/D2) between apomorphine and saline treated pigeons. Despite of the results of this experiment, suggesting a change in the dopaminergic receptors after chronic apomorphine administration, it is not possible to explain this change according to the pharmacological or conditioning hypotheses. It could be that pigeons learn to associate the home cage with the apomorphine effects. The next experiment (number 4) in this work is an exploratory one, searching for the physical place in the pigeon's brain where apomorphine could act, inducing the pecking stereotyped response. Cannuli were directly implanted into two brain areas; the nucleus accumbens septi and the caudal area of the striatum, including the paleostriatum primitivum and paleostriatum augmentatum. Once the cannuli were bilaterally implanted in the corresponding brain areas, apomorphine was injected: first, intramuscularly (to sensitise the pigeons) and then intracranially (to test for the possibility that some of these areas are able to maintain the pecking response level). The results of this experiment suggest that the nucleus accumbens (and not the caudal area of the striatum) is able to induce some apomorphine-like pecking. This result will be discussed later together with the results of experiments included in Chapter VII.

A second associative learning paradigm is presented in chapter V. It consists of a simultaneous visual discrimination task. Six different colours were simultaneously presented, only one being food-related. This discrimination task was set up in an attempt to induce a fast and consistent learning process. The design was based on the matching to sample experiments performed by Wright and Delius (1994) who used gravel stimuli. A total of six pots are simultaneously presented. Five contained different coloured grit (S-) and one coloured grit plus buried food (S+). Pigeons are required to dig in the grit to find the food. This way, the colour of the grit is associated with food presence. During the test, the same grit-containing pots that were presented during training are presented but without any food, and the choices and amount of pecking to S+ are recorded. Experiment 5 consisted of two phases. In the first of them two groups of pigeons are trained either to discriminate one of six coloured grits or

not to discriminate at all (every grit contained food). The results of this first phase revealed that pigeons can associate readily a given coloured grit with food. During tests they showed a very high preference for S+. Conversely, in the non-discriminative group, no preference for any particular stimuli (except for some spontaneous colour preference) was observed. In order to assess the degree of influence of both, a discriminative- and a non-discriminative task over a subsequent discriminative learning of a new different positive stimulus, both groups of pigeons underwent a second experimental phase. Here, they were trained to discriminate a second different positive stimulus (S2+). Compared to pigeons that were previously trained to discriminate a first positive stimulus, the results showed that it was harder for pigeons who had undergone a previous non-discriminative training to acquire the association of a new positive stimulus. These results are discussed in terms of relearning and latent inhibition processes.

To assess the dopaminergic and glutamatergic roles in associative and discriminative learning, the above mentioned coloured-grit-food association was used. In chapter VI the test of three different antagonist drugs using this associative paradigm were discussed. In experiment 6, the D2-like dopamine receptor antagonist haloperidol was used to test the hypothesis that D2-like dopamine receptors are closely involved in this associative and discriminative task. During training, two different doses of haloperidol were injected into two different pigeon groups. Conversely, a third group received only saline solution. Training consisted of the discrimination of a given S1+. During tests, a saline injection was given to all three groups. Results did not show any impairment in learning acquisition, suggesting that D2-like receptors do not mediate the acquisition of this discrimination task. Using a similar design, the role of D1-like receptors in this paradigm was assessed. Next, an extra group was added. It consisted of pigeons trained to discriminate a given S1+ under the effect of saline. In order to test for the role of the D1-like receptors in the retrieval of this associative learning, this group was then tested under the effect of SCH-23390. A drug and a saline solution were injected intracranially into the paleostriatum augmentatum and paleostriatum primitivum areas. Surprisingly, the group undergoing the saline administration exhibited a very low response compared with the responses of animals that perform the task without intracranial drug administration. This unexpected result accounts for the fact that pigeons show higher responses under the effect of the D1-like receptor antagonist, SCH-23390. In addition, no blocking effect on the acquisition and retrieval of this discriminative task was observed, suggesting that D1-like receptors are not involved in this task. Finally, experiment 8 assesses the role of the NMDA on the same discriminative task. MK-801, a NMDA receptor

antagonistm, was used in the same experimental design as for SCH-23390. The data shows that in the acquisition and retrieval of the learning of this discriminative task, the NMDA receptors do not play a role at all. The associative and discriminative tasks used in this work to assess the role of glutamatergic and dopaminergic neurotransmitters suggest the existence of more than one learning mechanism that could account for all experimental results.

Another associative learning paradigm was used to assess the role played by dopamine and glutamate on associative learning is the apomorphine-induced learning paradigm. Here, the same drugs employed for the assessment of the dopamine and glutamate role on the discriminative task were used. In our laboratory the apomorphine-context paradigm has been extensively studied. Nevertheless, only one experiment that tested for the role of the D2-like dopamine receptors in this paradigm has been done. In chapter VII, a design following an experiment performed by Godoy (2000) is presented. She assessed the effect of haloperidol in apomorphine-context conditioning. During the training, pigeons received haloperidol or apomorphine alone or apomorphine and haloperidol. During the test, all groups received saline in place of apomorphine as Godoy's design. Results suggest that haloperidol strongly impairs the acquisition of apomorphine-induced learning. This means that D2-like receptors are necessary for the acquisition of this associative learning, but not for retrieval processes. The role of D1-like receptors are also tested in experiment 10. To test the effects of SCH-23390 on the retrieval process, the same experimental design as in experiment 9, with the addition of an extra group was used. The results of this experiment support the hypothesis that D1-like receptors are involved in acquisition but not in retrieval processes. These experiments demonstrate that for this type of associative learning both D1- and D2-like receptors are necessary for acquisition but not for retrieval. To test the participation of NMDA in this associative learning, a similar experimental design was developed. The results of experiment 11 (where the NMDA receptor antagonist MK-801 was used) reveal that NMDA receptors are necessary for the acquisition as well as for the retrieval processes. These findings are then discussed in terms of the associative learning model proposed by Wickens (1990).

The last chapter (chapter VIII) discusses all experimental results presented. They are revised in the light of associative learning and in terms of how they fit Wickens associative learning model. The main conclusion is that the associative learning phenomena is a rather wide process in which several different mechanisms could be involved. While the associative and discriminative paradigms used in this work do not fit Wickens model, the apomorphine-induced learning does. Both paradigms share some characteristics (i.e., feeding behaviours and visual recognition of the stimuli). It was demonstrated that the apomorphine-induced

pecking corresponds to a different neuronal activity than the hunger-induced pecking does. The associative mechanisms between, the glutamate and dopamine systems in the striatum seem to be functional, at least for some forms of associative learning. Nevertheless, the glutamatergic and dopaminergic roles in additional associative learning paradigms should be further tested.

Chapter I

A general introduction

Through the centuries, human beings have endeavoured to understand themselves. In this everlasting effort, many have studied the way behaviours develop and the mechanisms underlying learning and memory. Various analytical approaches have given rise to disciplines such as philosophy, psychology and ethology. With time, it became clear that understanding behaviour, brain physiology, and the highest cognitive abilities could only come about through the integration of different disciplines, from psychology to biology, including the more recently formulated molecular neurobiology and computational neuroscience.

Biopsychology is a most recent and productive integration of several different psychological and biological approaches. Its foundation can be traced back to Charles Darwin's theories about the origin of species and the ascendance of man (1859), further developed by neo-darwinism and the re-discovery of Mendel's formal genetic (1866), and also with the more recent clarification of the molecular structure of genes by Watson and Crick (1953). It is currently thought that behaviour and cognition in humans and other animal species are products of the interactions among the molecular structures of deoxyribonucleic acids, which are capable of auto-replication in an imperfect way (Dawkins 1976). Nowadays, there is no doubt about the biological nature of behaviour. Even for the complex human being, whose psychology has traditionally been the focus of study, a biological basis of behaviour is accepted.

In recent years, research in biopsychology has produced a huge amount of experimental evidence, greatly advancing our knowledge of learning and memory. However, the nature of most of the mechanisms underlying these processes has not been completely understood. Comprehension of such biochemical and molecular mechanisms could induce new applications in clinical therapy and learning methods. Consequently, such knowledge would cause a general improvement in quality of life, including relief from pathologies such as Parkinson's disease, or mental disorders such as Alzheimer's disease or schizophrenia. Because of this enormous potential, neuroscience and the study of cognitive brain functions is an active and fast-developing area of research with a promising future.

Within this research field, pharmacology, anatomy and molecular biology have produced a lot of information about learning and memory. These disciplines have provided us with essential tools for the study of neural connections between brain areas and the different

molecules acting in the synapses. They have also inspired the study of the creation or modification of synapses occurring as result of experience which in turn modifies subsequent behaviour. The present thesis follows a pharmacological approach to the mechanisms underlying a certain type of learning, namely associative learning. The vast majority of behavioural and neurobiological experimental results have been obtained using animal subjects. The experiments presented in this thesis have been carried out with the domestic pigeon Columba livia. In the study of animal behaviour and learning, rats and pigeons have traditionally been preferred. Even when these animals possess specific characteristics, experimental evidence indicates that there are incontestable similarities in learning processes shared across rather different species, including humans. Such a convergence can be mainly explained by the fact that all animals' neuronal systems should work according to the same few principles since they evolved from a common ancestor. Also, in all species, learning efforts indicate a common goal: adaptation to the environment. Charles Darwin started this line of reasoning as an attempt to characterise not only the evolution of physical traits but also of psychological or mental abilities (Darwin 1872). In a changing environment, learning becomes an essential mechanism of adaptation and therefore, a trait susceptible to evolution.

Learning and memory

Organisms do not normally respond to their environment in a fixed and invariable manner. They vary their responses depending on contextual circumstances and their previous experience with the type of stimuli they encounter. The mechanisms by which they modify responses as a consequence of experience are referred to as learning and memory. Learning is the mechanism accounting for the acquisition of new knowledge and memory is the process responsible for the retrieval of acquired knowledge. Although no universally accepted concept of learning exists, the basic idea is that experienced events somehow change behaviours by modifying some structure and consequently, the organisation of our nervous system. Domjan (1993) for example, defines learning as: "an enduring change in the mechanisms of behaviour involving specific stimuli and/or responses that result from prior experience with those stimuli and responses" pp. 13.

In an attempt to facilitate the study of learning mechanisms, different forms of learning have been characterised. Repeated experience with a certain stimulus which elicits a reflexive response may induce the simplest forms of learning, namely habituation or sensitisation. Habituation occurs when a repeated stimulus presentation elicits a progressively weaker

response (provided that sensory adaptation and fatigue can be ruled out). Sensitisation occurs when the repeated experience with that stimulus evokes progressively stronger responses. Both processes allow organisms to adjust to their environment. However, environmental stimuli are normally not experienced in isolation: different stimuli may occur together, a certain stimulus can reliably predict another, etc. The ability to comprehend such relations and consequently, to modify ones response, represents an adaptive advantage. Classical conditioning is the simplest mechanism by which animals can learn to associate stimuli. Such associative learning allows subjects to modify their behaviour according to a sequence of events in their environment and to predict which stimuli tend to happen simultaneously.

Classical conditioning

Classical conditioning, also named Pavolvian conditioning, was first described by the Russian scientist Ivan Pavlov (1849-1936). The initial findings that led Pavlov to formulate his learning theories occurred accidentally while he carried out studies on the physiology of digestion in dogs. He established that food given to dogs elicits reflexive gastric and salivary secretions. Further, he observed that when the oesophagus was cut and externalised by means of a fistulae through the neck, so that food could not get into the stomach, the gastric secretion was still present, almost as much as in the case of animals without this dissection. He concluded that the stimulus producing the reflexive gastric secretion could not only be food in the stomach, but also food in the mouth. He considered the food in the mouth as an anticipatory or signalling stimulus. Later on, Pavlov found that those dogs undergoing this experimental procedure several times produced reflexive stomach secretion by just seeing the dish used for their daily food. He concluded then, that there must be two kind of reflexes: physiological reflexes, invariably performed by all individuals of a species in response to a certain stimulus, and other kinds of stimuli which he termed conditioned reflexes, performed only by certain individuals as a result of their particular experience. All Pavlov's dogs produced gastric secretions when food was present in their stomachs, but only those that had experienced the experimental procedure produced gastric secretions at the sight of the dish. Pavlov's conclusion was that if two stimuli are presented in a repeated and consistently paired manner, subjects eventually learn to associate them. If one of those stimuli (termed unconditioned stimulus (US)) elicits a certain unconditional response the second stimulus (conditioned stimulus (CS)) acquires the ability to elicit the same or a similar response. In summary, one of the stimuli, called the unconditioned stimulus (US), invariably elicits an

unconditioned response (UR), the other, called the conditioned stimulus (CS) does not normally elicit the same response in the animal. But after repeated paired presentations of CS and US, the CS starts eliciting a conditioned response (CR) similar to the UR. In the case of the dogs, the US was the presence of food in the stomach, which elicited gastric secretion UR, while the presence of the dish was the CS, which eventually elicited conditioned gastric secretion, CR.

The context as a CS

The CS usually used in classical conditioning procedures is a discrete stimulus, which is presented for a brief period of time with a clear beginning and a clear end. The use of discrete CSs has provided a great deal of information about this type of associative learning. However, in every situation, the above mentioned stimuli occur in the presence of background or contextual cues and consequently, associative learning often occurs in that context. Such cues can be visual, auditory or olfactory stimuli present in the experimental situation where the classical conditioning training takes place. The contextual cues may even play the role of CSs. After a certain US is repeatedly presented with the consequent appearance of its UR, within a certain context, the context itself begins to act as a CS, eventually eliciting a CR. This situation has been termed context conditioning. Contextual cues can be considered continuous because they are not presented with any time restrictions during the experimental procedure.

Drug action as a US

Clinical research on the action of several drugs such as morphine, heroine, alcohol, scopolamine, benzodiazepines and amphetamine, among others, has yielded some surprising behavioural results and has led to research on drug-induced learning. Many studies investigated the ability of drugs to induce tolerance or sensitisation, when a certain dose was repeatedly administered. Tolerance refers to a decrement, and sensitisation to an increment, in the efficacy of a given drug dose, as a consequence of repeated administrations. Morphine administration for pain relief is a typical example in which the development of tolerance can be observed. In contrast, some psychostimulants like apomorphine, amphetamine and cocaine are known to induce sensitisation.

In classical conditioning, the stimulus has to be of such a kind that it invariably elicits a response in the animal. Thus, the effects of certain drugs which reliably induce a particular

response in the organism may constitute a suitable US in classical conditioning training. When subjects repeatedly experience the effects of a drug inducing a certain response (UR) in the presence of other (for example, environmental stimuli), the latter (CS) eventually comes to elicit a similar response (CR).

One of the earliest observations of an association between the effects of a drug and contextual cues was reported by Pavlov (1927). He wrote about the observations of his colleague Dr. Krylov, who injected morphine into dogs to produce several strong symptoms such as salivation, vomiting, and sleep. After 5 or 6 consecutive daily administrations, the preparations for the injections alone were strong enough to induce these symptoms. Pavlov concluded that in that situation, the symptoms were the effect not of morphine acting directly on the brain area responsible for vomiting, but of all external stimuli preceding the injections that had become associated with its effects. Since then, several experiments on classical conditioning, using drug effects as the CS, have been carried out. Among the most recent are (Godoy & Delius 1999, Damianolopoulos & Carey 1994, Stewart 1992, Poulo & Cappel 1991, Stewart & Vezina 1988, Stewart & Eikelboom 1987). In rats, for example, amphetamine induces increased locomotion. When rats received repeated amphetamine injections in a certain experimental environment, they also showed increased locomotion with saline injections in that environment (Stewart & Vezina 1991). The potent dopaminergic agonist apomorphine elicits a stereotyped repetitive pecking response in pigeons which sensitises over repeated administrations of this drug. When pigeons receive apomorphine in a different context, the pecking sensitisation is no longer observed. In the present thesis, in order to study the possible participation of the glutamatergic and dopaminergic systems in learning, the classical conditioning association between apomorphine effects and contextual cues in pigeons will be used. Context-conditioning with apomorphine sensitisation will be further described in chapter III.

Discrimination learning

Generalisation and discrimination are two opposite phenomena in learning situations. After a conventional, paired US-CS presentation training, the CS (for example a 440 Hz tone) usually elicits a strong CR. Generalisation is said to occur when different CSs, for example tones of 500, 600, 700 or 800 Hz, still elicit some measurable CR. The greater the difference from the original CS, the weaker the CR it elicits. The response gradient in relation to the stimulus similarity gradient describes the generalisation in a certain situation. Discrimination denotes

the differential response of a subject to two similar stimuli. In a typical discrimination training, two similar stimuli are presented to the subject. One of them is paired with a reward and presented in half of the trials (the CS+ for example, a 440 Hz tone), while the second stimulus, presented in the remaining trials, is never followed by a reward (the CS- for example, a 500 Hz tone). In this situation, at the beginning, CR occurs after both the CS+ and the CS-. Nevertheless, with continued training, responses to the CS- gradually drop out. Eventually the animal will only respond to the CS+. In the opinion of Robert Bolles (1978) "just as generalisation is necessary for learning to be manifest in behaviour, so there must be discrimination if an animal is to respond intelligently to the world about it." In this thesis, the pigeons' ability to perform in a discrimination training has been used to develop a learning paradigm in which the possible participation of the glutamatergic and dopaminergic systems were assessed. Briefly, pigeons were confronted with several pots containing grit of different colours, only one of them containing food. They had to discriminate the food-containing coloured grit from the rest. This novel learning paradigm is discussed in chapter V in greater detail.

The experiments presented in this thesis investigate the role of dopamine and glutamate in pigeon learning, according to both the classical conditioning and the discrimination learning paradigms. Besides comparing results obtained with two different learning tasks, it was also possible to compare results between a drug-induced (the apomorphine-context conditioning) and a drug-free (the discrimination task) learning. A model of associative learning, proposed by Wickens in 1990, addressing the interaction of glutamatergic and dopaminergic systems on striatal neurones, offers a theoretical framework within which results of these experiments can be discussed.

Dopamine in learning

A well-established associative learning paradigm developed in our laboratory (Godoy & Delius 1999, Wynne & Delius 1995, Lindemblatt & Delius 1987, Basten-Kreft 1977) involves the dopaminergic system and consists of the association between the effects of the potent dopaminergic agonist apomorphine (Apo) and contextual cues in the domestic pigeon Columba livia. Apo is known to elicit feeding stereotypes in many different species, including humans (e.g. Glasgow & Ewert 1997, Szechtman et al. 1987, Fekete et al. 1970). In pigeons, an effective Apo injection elicits bouts of repetitive pecking, lasting for about one hour (Basten-Kreft 1977). When a certain Apo dose is repeatedly injected, the total induced

pecking response increases or sensitises with each injection up to a dose-dependent asymptotic level (Basten-Kreft 1977). The most simple explanation for such a sensitisation is the physical accumulation of the drug in the organism, which would cause the progressive response augmentation (Kalant 1998). In this case, longer inter-injection intervals should result in smaller sensitisation than shorter intervals. However, lengthening the inter-injection interval from the usual one day to three days, has been found to increase the magnitude of the sensitisation effect instead of reducing it (Keller, personal communication). Nevertheless, two other possible explanations for this drug induced behavioural sensitisation can be hypothesised. The first one assumes that circulating Apo directly produces pharmacological adjustments. These in turn, bring about a change in the efficacy of the relevant synaptic transmission, for example, through modifications in the number or affinity of the dopamine receptors. The second explanation is based on learning and assumes that circulating Apo would initiate learning processes that would indirectly modify the effectiveness of the relevant synapses. There is of course no *a priori* reason to assume that both types of mechanism must be mutually exclusive. Accumulating evidence supports the later account. Lindenblatt and Delius (1987) found that the Apo-induced pecking could be classically conditioned to a visually distinctive cage. Pigeons that repeatedly experienced Apo in a distinctive cage and saline in another one, pecked significantly more in the first than in the second cage when later tested under saline (without Apo). That is, Apo acted as unconditioned stimulus (US) which elicited an unconditioned response (UR pecking). When the US was repeatedly paired with a cage (conditioned stimulus CS), the CS elicited a conditioned response (CR pecking). More recent evidence (Keller & Delius 2001, Godoy & Delius 1999, Wynne & Delius 1995) also supports the hypothesis that the Apo-sensitisation is mainly (or even exclusively) due to the development of a CR pecking associated with a particular cage, which adds to the UR pecking elicited by the drug. However, possible non learning-mediated, pharmacological changes directly induced by Apo, which may influence the pecking sensitisation, cannot be confirmed or disregarded without adequate experimental evidence.

There are five different types of dopamine receptors denoted as D1, D2, D3, D4, and D5. Auto-receptors (of the types D2/D3) can be found on the pre-synaptic membrane (Langer 1997, Sibley et al. 1993). Based on some shared characteristics, they were classified as belonging to D1 and D2 families, or D1- and D2-like receptors. D1-like receptors include D1 and D5 with similar amino acid sequences, both stimulating the adenylate cyclase. The D2-like receptors include D2, D3 and D4 also with similar structure, all of them inhibiting the

adenylate cyclase. To my knowledge, no study has addressed the role of the different dopamine receptor types in the pecking sensitisation of pigeons. However, it is well known that both types of receptors are necessary to induce stereotypy (Beninger 1993).

The Wickens' learning model

The hypothesis on the participation of excitatory amino acids in the mechanism of learning and memory was proposed some years ago and widely accepted (Lee et al. 1998, Stecher et al. 1997). This hypothesis is mainly based on studies of the hippocampus in rats. However, this is not the only brain structure critically important to the formation and execution of learned reactions. The basal ganglia, including the striatum, also play an important role (Miller 1981). The main input from the cortex to the striatum comes through glutamatergic fibres. Additionally, micro-injections of glutamatergic preparations (agonists or antagonists) are capable of altering the animal's conditioned reflexes (Schmidt 1986). The dopaminergic system has also been extensively reported to participate in learning processes (Dearing & Branch 1981, Seeman 1981).

Dopamine has been found to be involved in several functions, including two higher-level brain functions: reward-mediated learning and motor activation. In both of them, dopamine appears to mediate a synaptic enhancement in the cortico-striatal pathway. The relationship between motor activation and learning is extremely complex. It is very difficult to measure behavioural parameters indicating the acquisition of learned responses without being influenced by performance. Behavioural evidence suggests that the interaction between motor activation and learning is a reflection of the way these two operations are related in the organisation of behaviour. The same or related brain components may thus be part of both the initiation of motor activity and the learning of a new response (Wickens 1990).

In reward-mediated learning, the ascending dopamine pathways in the forebrain might mediate the reward signal itself (Miller 1988). This kind of learning offers a framework for a neural model consisting of a number of alternative connections between a population of neurones which represents the stimuli and another population involved in selecting and initiating particular behavioural responses. In this model, reward-mediated learning consists of selecting and strengthening particular stimulus-response connections. Such a model implies the existence of a large number of alternative stimulus-response connections, over which a reward signal could exert control. It has already been proposed that the striatum possesses the necessary anatomical structure for such a mechanism (Miller 1981). In the striatum, cortical

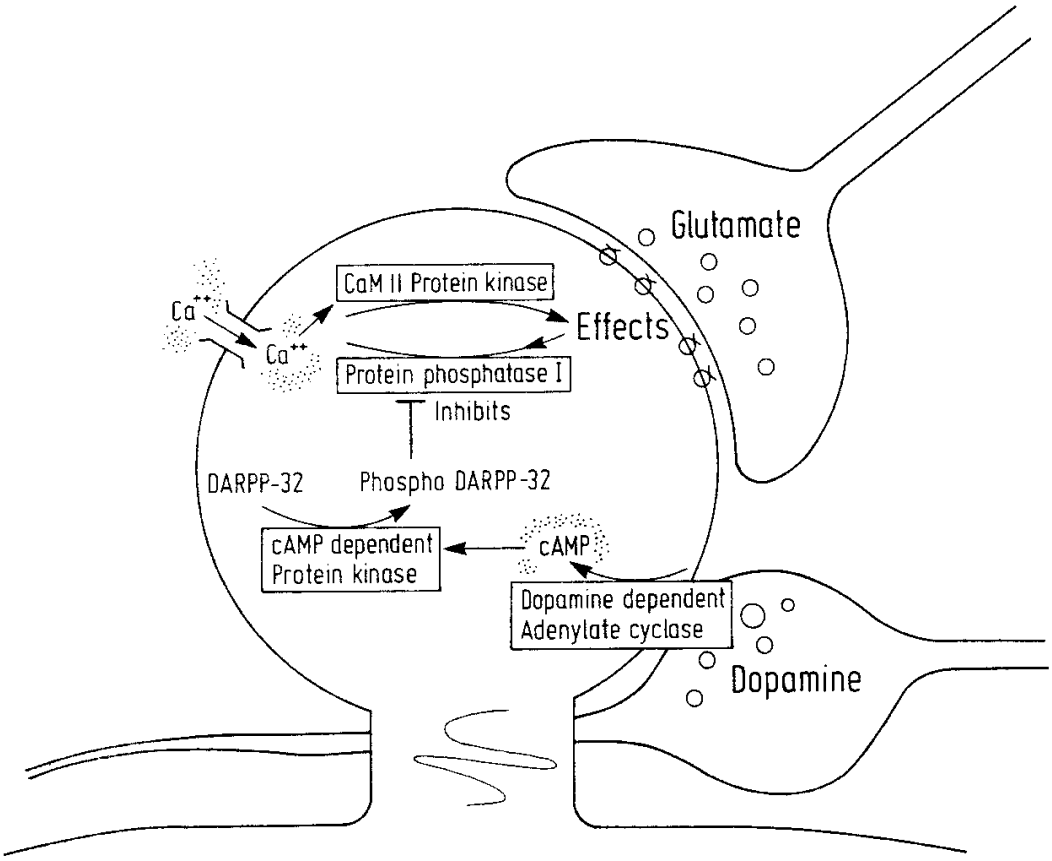


Figure 1.1. Wickens' postulated mechanism for synaptic enhancement in the striatum. Dopamine may exert a permissive effect on synaptic enhancement by inhibiting protein phosphatase I, thereby allowing the effects of a calcium-activated protein kinase to accumulate. The stimulation of the adenylate cyclase by dopamine induces the phosphorylation of DARPP-32, an effect mediated by the D1-like receptors. In this scheme, the elevated levels of spine calcium function as a "state of readiness" for enhancement by dopamine (taken from Wickens 1990).

and dopamine inputs terminate on the same population of neurones, often ending on a shared dendritic spine (Kubota et al. 1987, Freund et al. 1884). Furthermore, the dopaminergic system seems to modulate the glutamatergic synapses in the striatum through a Hebbian mechanism (Greengard et al. 1991).

It is known that reward-mediated learning involves the enhancement of synaptic transmission at selected subtypes of synapses. However, the main problem facing this connectionist model is that the relevant synapses may not be activated at the time of reward. Miller (1981, 1988) proposed that a "state of readiness" may be required, which consists of the temporary selection of particular synapses which have been effective in firing output neurones. Only such selected synapses would be eligible for reward-mediated learning. In this

situation, any synapse active in firing a striatal neurone would be strengthened. Some predict that once strengthened, this synapse will become more likely to be strengthened again. This positive feedback loop leads to repetitive activity in a limited range of responses (Wickens 1990).

The details of the model are summarised in figure 1.1. Striatal neurones with D1-like receptors are rich in a phosphoprotein known as dopamine-and-cyclicAMP-regulated-phosphoprotein 32 (DARPP-32). Phosphorylation of DARPP-32 is regulated by dopamine acting through cAMP, and may mediate specific interactions between dopamine and glutamate, acting through Ca^{2+}. In one possible sequence of events, glutamate may lead to the increment of calcium levels through depolarisation-sensitive channels, and thus to the activation of the calcium-dependent protein kinase. When phosphorylated, DARPP-32 inhibits protein phosphatase 1. Since protein phosphatase 1 reverses the effects of some of the protein kinases thought to be important in calcium-activated synaptic modification, the possibility exists that dopamine could exert permissive effects in this indirect way.

The potential mechanism for modifying synapses selected by raised calcium levels could work as follows. The activation of D1-like receptors could increase cAMP levels. The elevated cAMP activates in turn the cAMP-dependent protein kinase, which brings about the phosphorylation of DARPP-32. This in turn inhibits protein phosphatase 1. The consequent effect is the turn-off of the usual action of protein phosphatase, thereby enhancing the effects of protein kinases, such as calcium- and calmodulin-dependent kinase II (CaM kinase II).

The model of associative learning developed by Wickens offers a framework for the discussion of the implications of dopamine and glutamate in the learning process. This thesis will discuss the above model in light of results obtained with dopaminergic and glutamatergic drugs in pigeons, using two different learning paradigms.

The hypothesis

The main objective of the present work is to investigate the involvement of dopaminergic and glutamatergic systems in learning. Three different stages of the learning process are assessed: acquisition, establishment and retrieval. All three stages will be analysed in further detail in chapter III. The role of both neurotransmitter systems in learning is investigated in two different learning paradigms: the context conditioning of Apo pecking effects and the simultaneous discrimination of a food-associated colour, described in chapters III and V respectively. Besides those experiments directly related to the main objective of this thesis, other experiments are also presented. These help to describe and characterise the learning

paradigms and the function of dopaminergic cells in the nervous system of pigeons. Chapter II contains the results of an immunohistochemical exploration of the dopaminergic system in the spinal cord of pigeons. Until now, dopaminergic cells have never been found in the spinal cord of adult birds.

Chapter II:

The glutamatergic and dopaminergic systems.

The glutamatergic system

Glutamate based transmission

During the last 30 years two classes of amino acids that occur in high concentration extracellularly and have profound effects on central nervous system function have been intensively investigated. These are the so-called “fast transmitters”: the inhibitory amino acids glycine and **γ-a**mino**b**utyric **a**cid (GABA), and the excitatory amino acids L-glutamate and L-aspartate.

To produce fast neuronal excitation, the vast majority of synapses in the vertebrate central nervous system employ the excitatory amino acids L-glutamate and L-aspartate as neurotransmitters (Fonnum 1984). The suggestion that L-glutamate and L-aspartate may function as neurotransmitters was first made by Hayashi (1954), who observed that a topical application of these amino acids to the rodent cerebral cortex causes cortical seizure activity. Later, Curtis et al. (1959) found that L-glutamate and L-aspartate induce depolarisation of neurones in the spinal cord resulting in an increase of action potentials. Biochemical and electrophysiological studies indicate that L-glutamate and L-aspartate are present in relatively high concentrations in the brain, are released in a calcium-dependent manner upon membrane depolarisation, and have a high-affinity uptake system that rapidly removes then from the synaptic cleft (Fonnum 1984).

Up to 80% of the synapses in the human brain may use L-glutamate as their neurotransmitter and this is reflected in the dominant release of L-glutamate from cerebrocortical synaptosomes which exceeds by a factor of eight that of the next most abundant transmitter, GABA (Nicholls 1995). It is also widely accepted that L-glutamate as the predominant excitatory transmitter in the mammalian and other vertebrates central nervous system acts at a range of different receptors types (Monaghan et al. 1989, Fonnum 1984).

Glutamate synthesis

L-glutamic and L-aspartic acids are flexible and versatile molecules. They exist in several conformations and can be obtained by metabolising other molecules; this is probably a major factor in their ability to activate a range of different excitatory amino acid receptors (Watkins et al. 1990). Specificity for receptor subtypes may be achieved by restricting the conformational variability to that appropriate for interaction with a particular receptor, and/or by introducing substituent groups into positions that hinder attachment to certain subtypes, while still permitting appropriate interaction with others. Ring structures within the glutamate molecule can introduce both features at once (Watkins 1991).

L-glutamic acid is synthesised in nerve terminals from i) glucose via the Krebs cycle (glucose is partially oxidised to pyruvic acid to obtain energy; the Krebs cycle completes the oxidation by metabolising the pyruvic acid to carbon dioxide and water) and transamination (transamination is the metabolic process in which an amine group is transfered from one molecule to another *via* the activity of an enzyme) of α–ketoglutarate and ii) glutamine which is synthesised in glial cells, transported into nerve terminals and locally converted by glutaminase into glutamate. In glutamatergic terminals, L-glutamate is stored in synaptic vesicles from which it is released by a calcium-dependent exocytotic process. Termination of the action of glutamate in the extracellular space is achieved *via* high and low affinity neuronal and glial re-uptake processes. In the glial cells glutamine synthetase converts L-glutamate into glutamine, which is transported into the neighbouring nerve terminal (*via* a low affinity process) where it serves as a precursor for glutamate. Glutamine can also be oxidised in the astrocytes into α–ketoglutarate (*via* the Krebs cycle) which can be actively transported into neurones to replace the α–ketoglutarate lost during the synthesis of neuronal glutamate (Carter et al. 1986). A schematic model of the L-glutamate nerve terminal and glial cell is shown in figure 2.1.

Glutamate receptors

The synaptic responses elicited by the excitatory amino acids L-glutamate and L-aspartate in the vertebrate's CNS are mediated by distinct groups of receptors which have been categorised as ionotropic or metabotropic on the basis of pharmacological, electrophysiological and biochemical studies (Young et al. 1995, Sommer & Seeburg 1992, Nakanishi 1992, Monaghan et al. 1989). Figure 2.2 illustrates the glutamate receptor family. The metabotropic receptors are

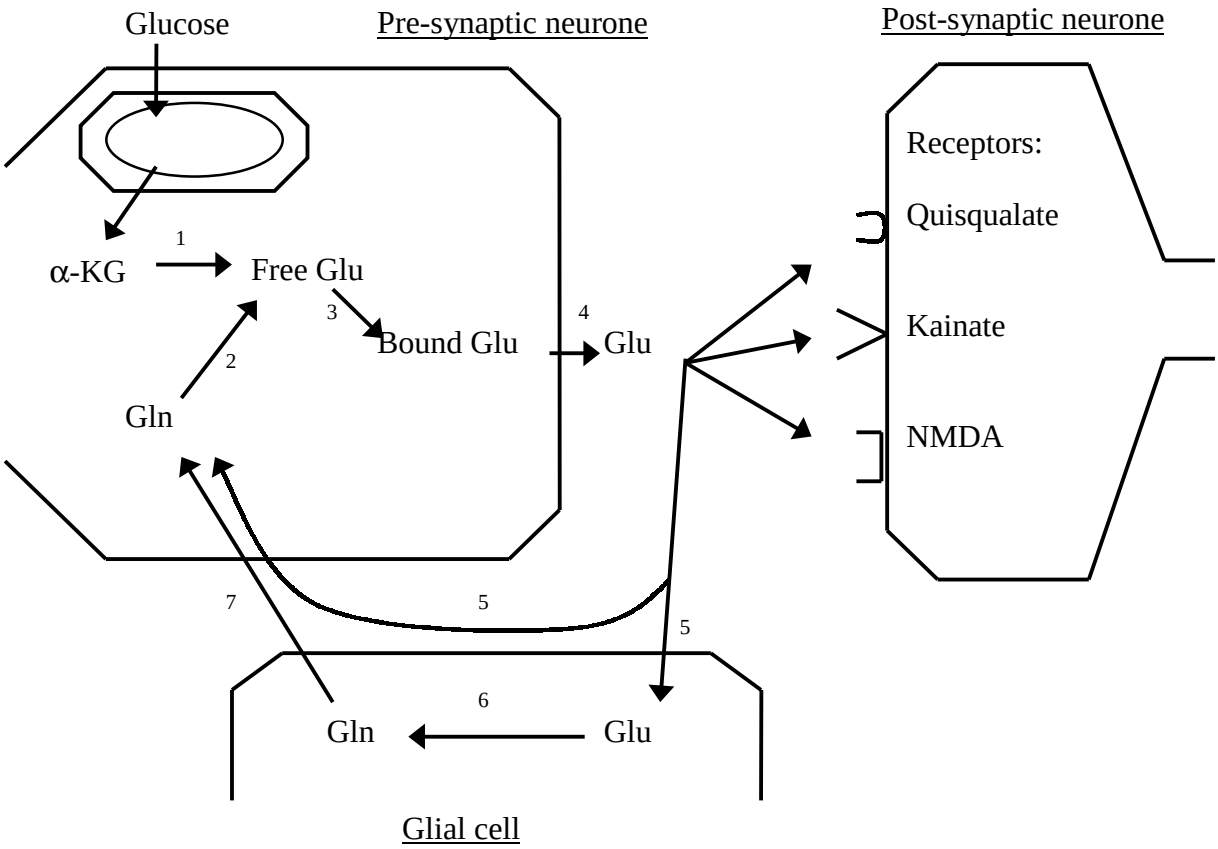


Figure 2.1. Schematic model of glutamatergic nerve terminal, postsynaptic process and glial cell: 1) α-ketoglutarate (α-KG), derived from the oxidation of glucose within mitochondria, is converted to L-glutamate (GLU) by the enzyme α-ketoglutarate transaminase; 2) phosphate-activated glutaminase catalyses the conversion of glutamine (GLN) into L-glutamate; 3) L-glutamate is stored in synaptic vesicles; 4) L-glutamate is release from the nerve ending in a calcium-dependent fashion for the interaction with postsynaptic receptors; 5) L-glutamate in the extracellular space is transported by a sodium-dependent process into glutamatergic nerve terminals and glial cells; 6) glutamate in glial cells is converted into glutamine by the action of glutamine synthetase; 7) glutamine in glial cells is transferred back into glutamatergic nerve endings (Palmer 1991 modified).

coupled to phospholipase C or adenylate cyclase (*via* pertussis toxin sensitive G proteins) and control the production of intracellular messengers, whereas the ionotropic receptors contain integral cation-specific ion channels. According to pharmacological and biochemical studies, glutamatergic receptors can be distinguished in ***N*-methyl-D-aspartate** (NMDA), kainate and α-**a**mino-3-hydroxy-5-**m**ethylisoxyzole-4-**p**ropionic **a**cid (AMPA) receptors for the ionotropic

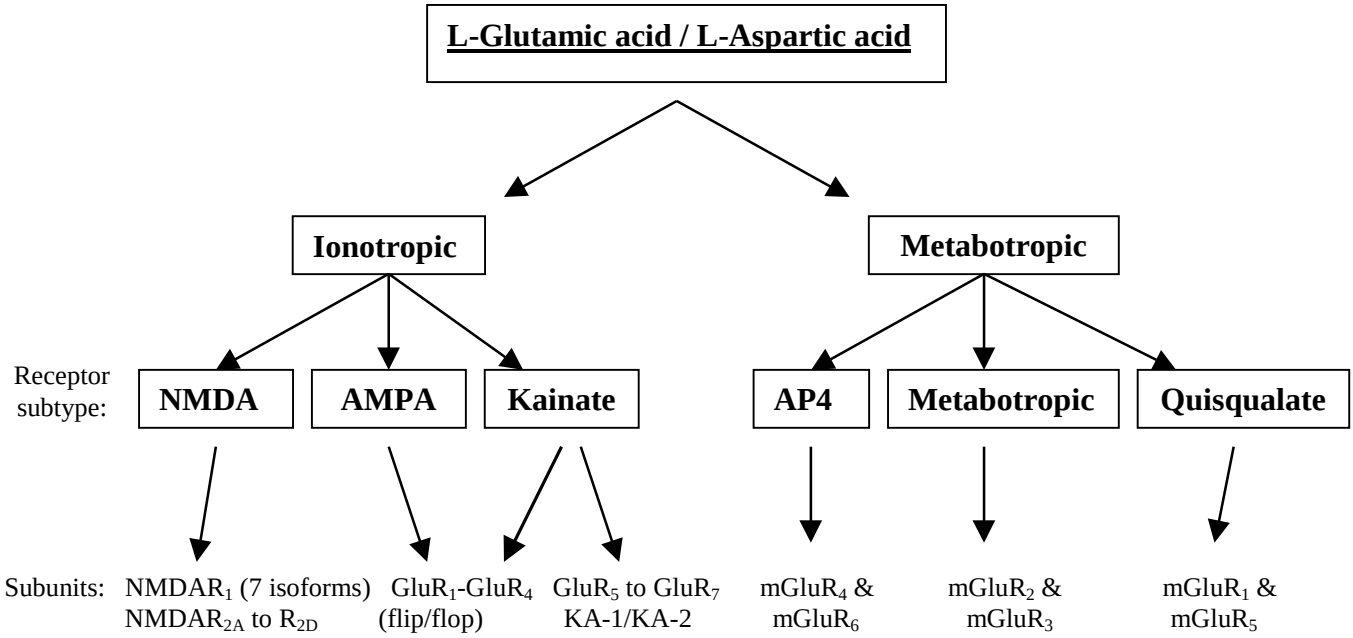


Figure 2.2. Excitatory amino acid receptor classification. AMPA and kainate receptors share some of the protein sub-units. These receptors together with NMDA are the ionotropic glutamate receptors. AP4 (or mGlu III), metabotropic (or mGlu II) and quisqualate (or mGlu I) are the metabotropic glutamate receptors (Scatton 1993, modified).

family receptors (Scatton 1993). Several splicing variants for the glutamate metabotropic receptors (mGlu) involve eight different mGlu, divided into three groups on the basis of homology between amino acids sequences, but also in terms of their pharmacology (Nakanishi 1992). The group I is activated potently by quisqualate (quisqualate receptor or mGlu I), and group II and group III (mGlu II, mGlu III or AP4 receptors respectively) are activated by (2-(2-**c**arboxy**c**yclopropyl)**g**lycine CCG-1 and **L**-2-**a**mino-**4**-**p**hosphonobutanoate (L-AP4) respectively. It is known that ionotropic receptors act in post-synaptic excitatory responses, while the mGlu can be located in both, pre- and post-synaptically (Salt & Herrling 1995). All glutamatergic receptors have already been cloned. Molecular cloning experiments have revealed a further heterogeneity. The AMPA-kainate receptors control a cationic channel permeable to sodium and potassium, while the NMDA receptor controls a cationic channel permeable to these ions as well as calcium (Sommer & Seeburg 1992). The three groups of metabotropic glutamate receptors (mGlu) are coupled to different second messengers pathways. The mGlu I ($mGlu_{1\ and\ 5}$) is coupled (*via* protein $G_{q/11}$) to the metabolism of phosphoinositol, mGlu II ($mGlu_{\ 2\ and\ 3}$) is coupled (*via* Protein G_i/G_o) to the adenylyl cyclase and inhibits the formation of **c**yclic **a**denosine-**m**ono**p**hosphate (cAMP), the mGlu III ($mGlu_{4,6,7\ and\ 8}$), like mGlu II, also inhibits the adenylyl cyclase, differing however, from mGlu II in its

pharmacology, since the glutamatergic agonist L-AP4 is only active in this mGlu III and not in the mGlu I and II.

NMDA receptors, which are more central in this thesis, comprise the assembly of two distantly sequence-related sub-units, at least one of seven different NMDAR1 sub-units (NR1A to 1G) and an NR2 sub-unit, of which four types (NR2A to 2D) have been characterised (Ishii et al. 1993, Sugihara et al. 1992), probably implying the existence of at least four NMDA receptor subtypes. While the NR1 sub-units are generated by alternative splicing of a single gene, the NR2 sub-units are the product of four highly homologous genes. The mature sub-units of NMDA receptors contain four hydrophobic sequence regions. It is expected that these sequences cross four times the lipid bilayer whose sub-units have extracellularly located amino- and carboxy-termini (Seeburg et al. 1994). NMDA receptors differ from the non-NMDA types of ionotropic glutamate receptors in several properties. These properties comprise a large single-channel conductance, high Ca2+ permeability, voltage-dependent Mg2+ block, slow gating kinetics, and a requirement for glycine (Seeburg et al. 1994). Some of these properties differ substantially, depending on which of the four modulatory NR2 sub-units assembles with the principal NR1 sub-unit. These data, combined with the expression characteristics of NR2 genes in the developing and the mature CNS, indicate that in many cell populations NMDA receptor properties differ. The data is consistent with the notion that early and late NMDA receptors may serve different physiological functions (Seeburg et al. 1994).

Glutamatergic pathways

Most experiments concerned with the glutamatergic pathways are performed using autoradiographic measurement, electrophysiological and pharmacological techniques and lesion of anatomically defined pathways in the mammalian brain. However, there are some equivalencies between the different excitatory amino acid pathways in pigeon and rat brains (Cuénod et al. 1981). For example, lesions of retino-tectal neurones, with kainic acid injections in the optic tectum, are similar in rats and pigeons; in both cases the kainic acid induced-histotoxicity is dependent in some co-operation between kainic acid and a population of optic fibres. Other works employing radioactive glutamine (precursor of glutamate and GABA) in rats and pigeons determined the release of glutamate (and GABA) in various nervous system structures in rat and pigeon brains. These data show that the release of glutamate is high in the rat striatum, medium in the rat and pigeon hippocampus and low in the rat cerebellum,

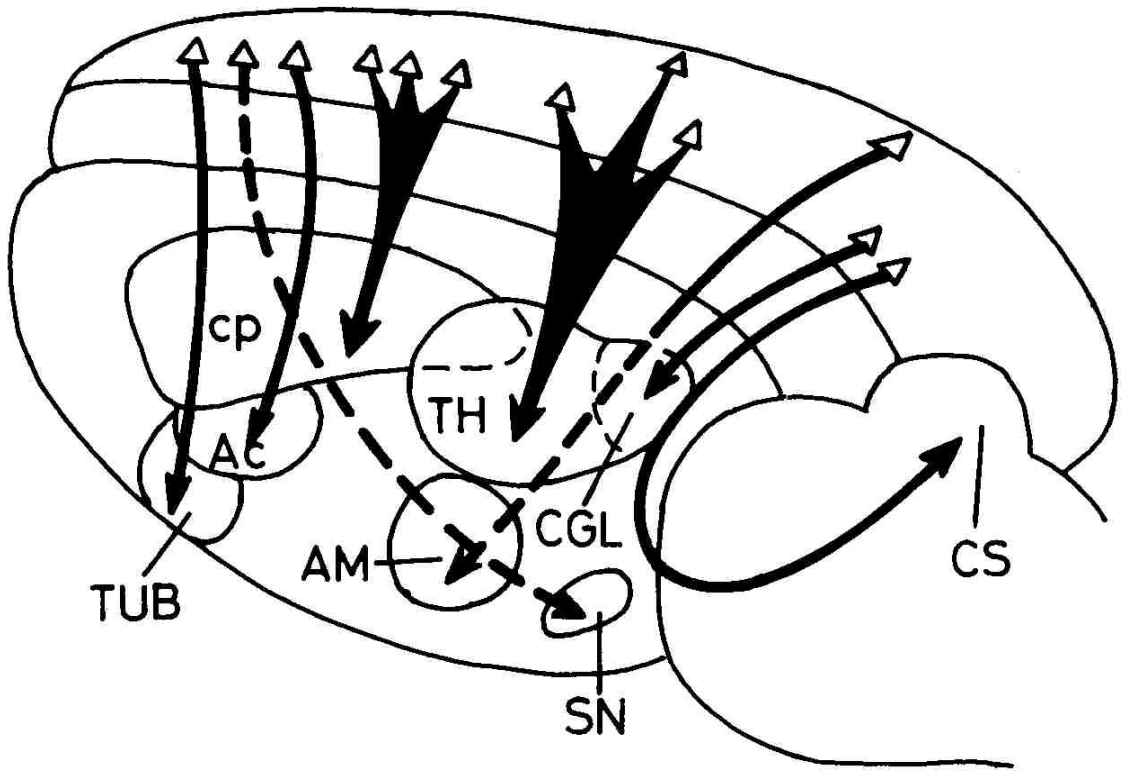


Figure 2.3 The origin and distribution of the glutamate fibers from neocortex. Ac, nucleus accumbens ; cp, neostriatum; TH, thalamus; CGL, lateral geniculat body; CS, colliculus superior; SN, substantia nigra; AM, amygdala; TUB, olfactory tubercule. The projection to AC, TUB and cp come mainly from the frontal part of the cortex. The projection to SN is very small and also comes from the frontal part. The projection to TH comes from the entire cortex, but particularly from pyriform cortex. The projection to AM also passes through the pyriform cortex. The projection to CGL and SC comes mainly from the visual cortex (taken from Fonnum et al. 1981).

substantia nigra and cochlear nucleus, as well as in the pigeon paleostriatum, cerebellum and optic tectum. Most of the glutamatergic pathways are descending: they originate in the neo- and allo-cortex and innervate most of the sub-cortical and spinal cord. The functionally most important glutamatergic pathways are the cortico-striatal, the thalamo-cortical pathway, the prefrontal or hippocampal pathway (which originates in the enthorinal cortex and projects to the hippocampus), the tectofugal or corticofugal pathway (which originates in the retina ganglion neurones and project to the contralateral optic tectum) and the cerebellum systems. Most of the cortico-cortical connections and primary afferent terminals to the spinal cord also use glutamate as a neurotransmitter (Scatton 1993).

Spinal cord afferent pathway: Studies using extra-cellular recording and iontophoresis *in vivo,* suggest that the mono-synaptic excitation of motor neurones and Renshaw cells is primarily mediated by non-NMDA receptors. There is also an NMDA receptor component to the mono-synaptic excitatory post-synaptic potential, recorded intra-cellularly (Pinco & Lev-Tov 1993), whereas poly-synaptic excitation is mediated by NMDA receptors (Davies et al. 1982). Subsequent *in vitro* studies broadly confirm these findings (Long et al. 1990). Additionally, other work has shown that in the spinal cord, a depressant pre-synaptic action is mediated by mGlu II and III (Pinco & Lev-Tov 1993).

Thalamo-cortical pathway: These system, involve the thalamic relay nuclei which receive a particularly sensory input and project to specific areas of the cerebral cortex (Jones 1985). The lateral geniculate nucleus, a specific thalamic relay of the visual system, can be stimulated by excitatory amino acids. The visually elicited excitation of lateral geniculate neurones can be reduced by both NMDA and non-NMDA antagonists (Sillito et al. 1990). Haldeman et al. (1972) found that the activation of the **v**entro**b**asal thalamus (VB) neurones by electrical stimulation of a peripheral nerve could be blocked by non-selective glutamatergic antagonists. Subsequent *in vitro* experiments demonstrated that both, NMDA and non-NMDA receptors are involved in the synaptic excitation of VB neurones by somatosensory afferents (Salt & Eaton 1991). Immunocytological studies suggest the participation of the mGlu receptors in the thalamic sensory transmission to be linked to the presence of mGlu I (Martin et al. 1992). It is also suggested that NMDA and mGlu I receptors may have a synergetic functional activity (Eaton & Salt 1990).

Cortico-cortical system: Experiments using electrodes in the motor cortex area indicate that thalamo-cortical excitatory post-synaptic potentials may be mediated predominantly by non-NMDA receptors. Interestingly, the recurrent excitatory post-synaptic potentials which could be elicited in pyramidal tract neurones by stimulation of the pyramidal tract are more sensitive to NMDA than to non-NMDA receptors (Salt et al. 1995).

Cortico-fugal pathway: The target areas of cortico-fugal systems include the thalamus, spinal cord, dorsal column nuclei, red nucleus, striatum and superior colliculus. Several studies have provided evidence for both NMDA and non-NMDA receptors involvement in these pathways (Davies et al. 1994). *In vitro* work on the rat striatum indicated that the cortico-striatal excitatory post-synaptic potentials may also involve NMDA receptors, even though the experiments that have been carried out *in vivo* did not reveal this component (Cherubini et al. 1988). It has been also demonstrated that the cortico-striatal excitatory postsynaptic potentials can be inhibited pre-synaptically by L-AP4, acting upon the mGlu III receptors (Calabresi et al.

1992). The cortico-thalamic pathway not only projects onto the thalamic relay neurones, but also onto intrinsic Golgi thalamic interneurones, and neurones of the thalamic reticular nucleus (Jones 1985). Both types of cells inhibit the thalamic relay neurones. Thus, electrical stimulation of the cortex can evoke both, excitatory and inhibitory effects on thalamic neurones.

Cerebellar systems: There is general agreement that cerebellar Purkinje cells have an unusual response profile to excitatory amino acids. There is good evidence that these cells possesses few or no NMDA receptors in the adult, but that the NMDA receptors are transiently expressed during development (Krupa & Crépel 1990). The non-NMDA and mGlu receptors (pre- and post-synatpically) are present in these pathways also in adulthood. There are three pathways with terminals in the cerebellum which are likely to use L-glutamate or L-homocysteate: a) the mossy fibers input to cerebellar granule cells, b) the parallel fibers from granule cells to dendrites of Purkinje cells, and c) the climbing fibers input onto the somata of Purkinje cells.

The hippocampal pathway: Includes the piramidal cells CA1, CA3 and others where most of the studies on NMDA receptors were performed. This piramidal cells receive input from different cerebral nuclei, for example, the Schaffer collateral-comisural inputs to CA1, medial and lateral perforant path inputs to dentate gyrus, and perforant path to CA3 piramidal cells. Additionally to the NMDA antagonists, AMPA and kainate are also effective to depress the excitatory post-synaptic potentials (EPSP).

The cortico striatal pathway: The striatum receives a major projection from the cerebral cortex. Some evidence suggests that most of this input is glutamatergic. However, the NMDA seems to have little effect on the cortical input to the striatum, since NMDA specific antagonist failed to depress cortically evoked EPSPs. These EPSPs are effectively depressed by non-NMDA antagonist like AMPA and kainate receptors, but the NMDA antagonist were effective to depress the EPSP in cells depolarised above –50mV or in Mg^{2+} free medium.

The physiological implications of the ionotropic and metabotropic excitatory amino acid receptors are too complex to be understood in a single or simple way. However, the glutamate receptors are apparently involved in synaptic transmission itself in addition to other proposed roles in phenomena such as learning and memory, development, and synaptic plasticity (Rison & Stanton 1995, Herrling 1987) as well as in a number of pathological conditions (Rison & Stanton 1995, Herrling 1989). Both NMDA and non-NMDA receptors (ionotropic receptors) are involved in all the processes mentioned below. The metabotropic receptors appear,

nevertheless, to be more linked only to synaptic plasticity and to have some role in the development of the CNS (Salt & Herrling 1995).

LTP and glutamate NMDA receptors

Synaptic plasticity is one of the major requirements for associative learning and memory. Some neurones have been shown to have plastic capacities. In 1973, Bliss and Lømo demonstrated that a brief high-frequency train of stimuli to any one of the three afferent pathways to the mammalian hippocampus produces an increase in the excitatory synaptic potential in the post-synaptic hippocampal neurone which can last for hours, days and even weeks. They called this facilitation **l**ong-**t**erm **p**otentiation (LTP). Later studies demonstrated that LTP can be either associative or non-associative. One of the most interesting properties of LTP is that LTP is specific to the active pathway. LTP itself seems to fulfil the Hebb's synapses, since it requires the post-synaptic depolarisation coincidentally with the activity in the pre-synaptic neurone. Then, it is possible to have the strengthening of a tetanus stimulation of a weak input, incapable of inducing LTP by itself, which co-occurs with an strong tetanus stimulation, capable of generating LPT on the same post-synaptic cell. The weak input is then potentiated. It is important to point out that this associative induction of LTP only occurs when both inputs are activated at the same time in the same post-synaptic cell. The LTP mechanism involves the glutamatergic receptors. A model for the induction of LTP is given in figure 2.4. The NMDA and the non-NMDA receptor channels are in post-synaptic membrane. In a normal glutamatergic synapse, glutamate is released from the pre-synaptic terminal and binds both NMDA and non-NMDA receptor channels, however only the non-NMDA are gated by Na^+ and K^+, while NMDA remains blocked by Mg^{2+}. When the post-synaptic membrane is depolarised by the action of the non-NMDA receptor channels, as occurs in a high-frequency stimulation, the depolarisation relieves the Mg^{2+} blockade of the NMDA channel. The NMDA channel then allows that Na^+, K^+ and Ca^{2+} flow, the entrance of Ca^{2+} to the cell triggers Ca^{2+}-dependent kinases (e.g. Ca^{2+}/Calmodulin kinase and protein kinase C). These kinase are thought to become persistently active. The maintenance of the LTP requires, in addition, an increase in the pre-synaptic transmitter release. Because of that, some message must be sent to the pre-synaptic neurone (nitric oxide or araquidonis acid metabolites are thought to be the retrograde messenger) that acts on the kinases of the pre-synaptic terminal and induces the increase of the neurotransmitter release.

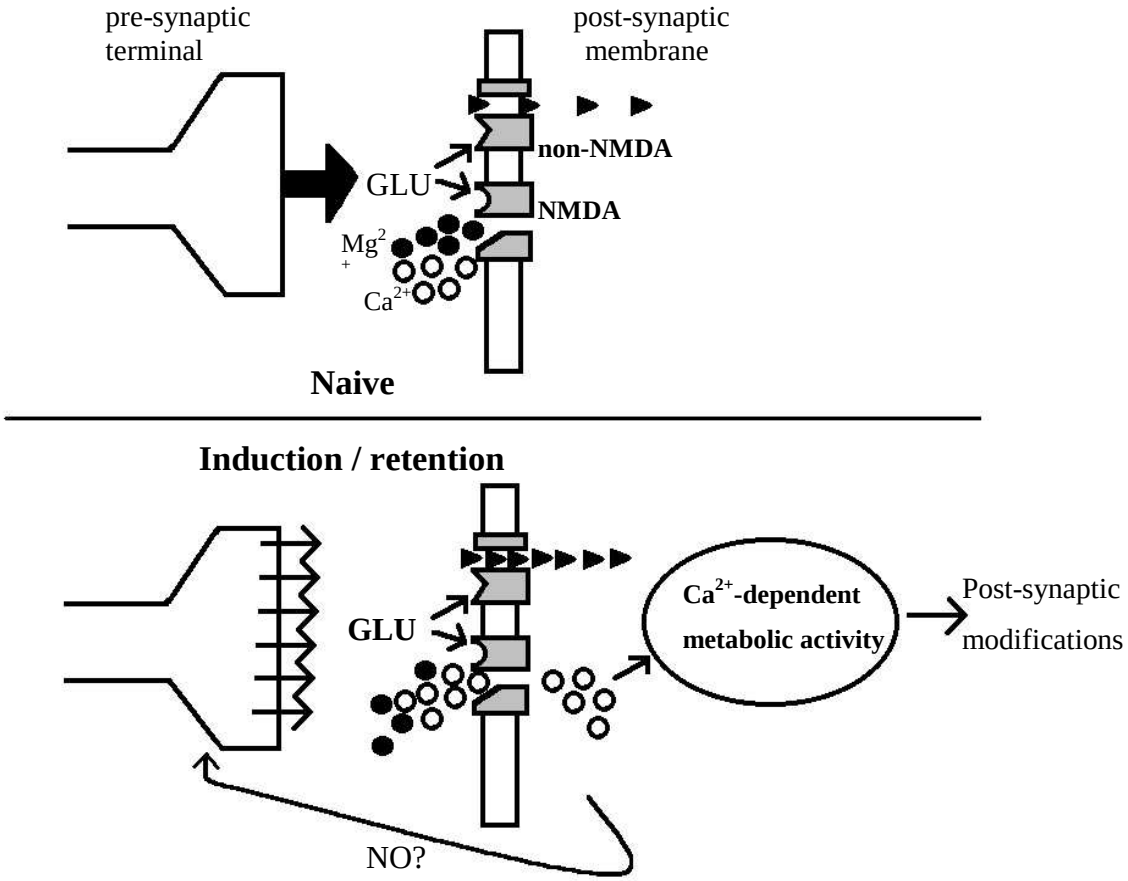


Figure 2.4 Schematic diagram of the role of NMDA receptors and intracellular Ca^{2+} (open circle) in the induction and retention of LTP. The upper part represent a naive situation. Single shock evokes the releases of glutamate which activates post-synaptic glutamatergic ionotropic non-NMDA receptors (filled triangles). In these conditions, NMDA receptors are blocked by Mg^{2+} (filled circles) and are not opened by glutamate. Tetanic stimulation of the pre-synaptic membrane leads to the induction of LTP. The Mg^{2+} block of NMDA is relieved (probably due to the depolarisation generated by the accumulation of glutamate in the synaptic cleft) allowing Ca^{2+} to flow through the NMDA receptors into the post-synaptic membrane. It also depolarised the membrane to the threshold for voltage-gated Ca^{2+} channel opening. The rise in intracellular Ca^{2+} concentration, thus produced in the post-synaptic element, triggers the subsequent events leading to the induction of LTP of the inotropic non-NMDA-mediated excitatory stimulation of the post-synaptic membrane. The metabolic Ca^{2+}-dependent processes lead to a lasting changes that enhance the response to the neurotransmitter. The nitric oxide (NO) could also serve as a retrograde messenger that regulate the glutamate release. The activation of metabotropic receptors would up-regulate NMDA receptors (no showed in the scheme).

Pharmacology of NMDA receptors

NMDA receptors at which NMDA and quinolinic acid are selective agonists, are ionotropic excitatory amino acid receptors. Actually, it is a receptor complex (Moriyoshi et al. 1991)

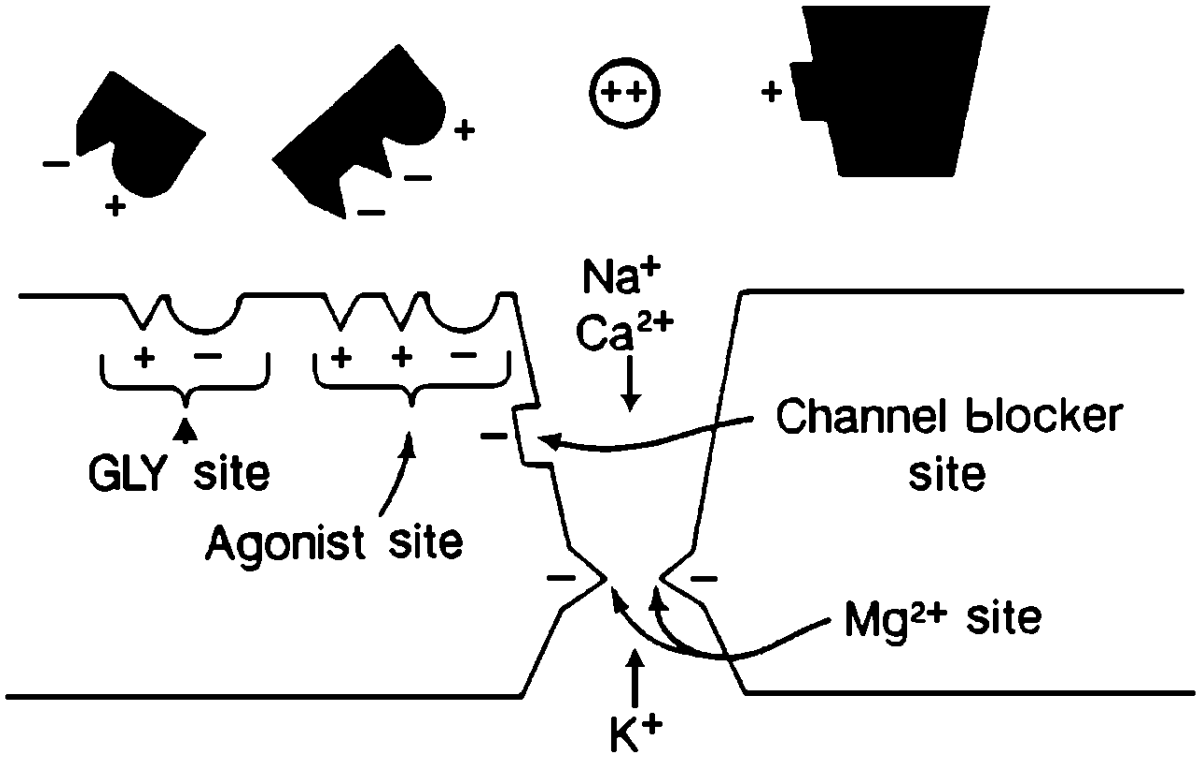


Figure 2.5. Schematic representation of the NMDA receptor cannel. The agonist site recognises the glutamate and other agonists. The glycine (Gly) site is a modulatory site. The channels blocker sites recognise MK-801, PCP and ketamine (taken from Watkins 1991).

comprising two agonist recognition sites and a site for the co-agonist glycine (Thomson 1990). Furthermore , polyamines interact allosterically with the NMDA receptor. Binding sites for the non-competitive NMDA antagonist phencyclidine (PCP) and related compounds (Meldrum 1991), as well as binding sites for magnesium (Collingridge & Watkins 1994) and zinc ions (Yeh et al. 1990) have been identified. The polycyclic amine, 5-methyl-10,11-dihydro-*5H*-dibenzo[*a,d*]cyclohepten-5,10-imine (MK-801, dizocilpine) is a very potent non-competitive NMDA antagonist which acts as an open channel blocker (Iversen 1994). The anaesthetics PCP and ketamine compete with MK-801 for the same binding sites. MK-801, ketamine and PCP have shown that the antagonist effect is agonist dependent, supporting the view that these compounds bind to a site within the ion channel of the NMDA receptor (Iversen 1994) as shown in figure 2.5.

MK-801 has been extensively studied in a number of animal models of epilepsy (Meldrum 1991), schizophrenia (Fendt et al. 2000, Koch et al 2000a) and ischemia (Iversen 1994) and it is very effective for reversing the symptoms in most of these models. In the same

way, this kind of animal model of human disease helps to prevent the adverse effects of MK-801 and PCP and to improve treatment in human clinics (Lodge 1992). Another finding was that MK-801 bound preferentially the activated form of the NMDA receptor: it seems that MK-801 needs to have the receptor channel activated by glycine and glutamate (Foster & Wong 1987).

NMDA receptors are also competitively and effectively blocked by a number of phosphono amino acids, among them are the (D)-2-amino-5-phosphonopentanoic acid ((D)-AP5), (D)-2-amino-7-phosphonoheptanoic acid ((D)-AP7) and (D)-3-(2-carboxypiperazin-4- yl)propyl-1-phosphonic acid ((D)-CPP) (Collingridge & Watkins 1994).

The dopaminergic system

Dopaminergic based transmission

Dopamine is one of the major neurotransmitters in the vertebrate central and peripheral nervous system. It is a catecholamine, which is a group of the monoamines. Its name is derived from its chemical name, **d**ihydr**o**xy**p**henylethyl-**amine**. There are three catecholaminergic transmitters in the vertebrate nervous system. They are dopamine, noradrenaline and adrenaline. All of them are synthesised from a common biosynthetic pathway using the amino acid tyrosine as a precursor (Figure 2.6). The conversion of tyrosine to the intermediate L-dihydroxyphenylalanine (L-DOPA) by the enzyme tyrosine hydroxylase is the rate-limiting step. L-DOPA is subsequently converted to dopamine, the first catecholaminergic neurotransmitter in this pathway. Further steps in the synthesis yield noradrenaline and adrenaline. Not all cells releasing catecholamines express the five enzymes involved in this pathway. Whether a neurone will release dopamine, noradrenaline or adrenaline depends on the enzymes of the catecholaminergic biosynthesis expressed in that particular cell.

Dopamine is stored in vesicles inside the synaptic terminals of the dopaminergic neurones. It is released from the vesicles to the synaptic cleft after a membrane depolarisation caused by an action potential. Dopamine molecules can diffuse and attach to their specific receptors on the post- and pre-synaptic membrane. Post-synaptic receptors mediate the action of dopamine on the post-synaptic cell while pre-synaptic receptors, called auto-receptors, mediate a regulatory feedback on the dopamine release. Further regulatory mechanisms include the re-uptake of dopamine molecules into the pre-synaptic neurone and the action of the

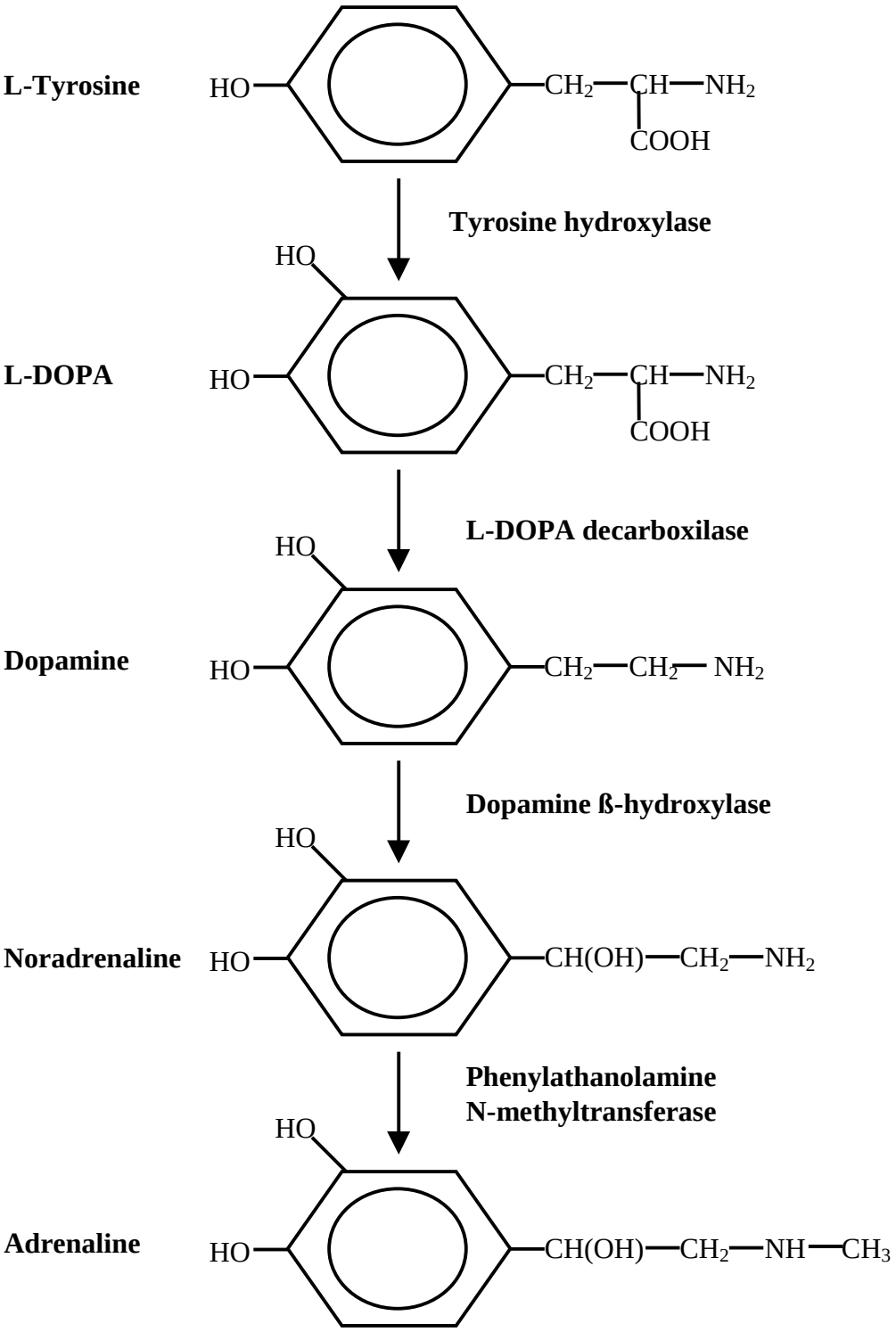


Figure 2.6. Biosynthetic pathway of the catecholaminergic neurotransmitters. The figure shows the conversion of tyrosine to L-dihydroxyphenylalanine (L-DOPA) by the enzyme tyrosine hydroxylase. This is the rate-limiting step. L-DOPA is converted to dopamine by the dopa-decarboxylase. Further steps in the synthesis are the conversion of dopamine to noradrenaline by the dopamine-β-hydroxylase and the conversion of noradrenaline to adrenaline by the phenyletanolamine-N-methyltransferase. Whether a neurone will release dopamine, noradrenaline or adrenaline depends on the presence of the relevant enzymes within the cell (taken from Blaschko 1973).

enzyme monoamine oxidase (MAO) present in the synaptic cleft, which catabolises free dopamine molecules. Figure 2.7 shows a scheme of the dopaminergic synapse.

Dopamine receptors

Dopamine receptors are proteins in the cellular membrane. There are different receptor types. These receptors are mainly coupled to two different effector systems via guanine nucleotide regulatory proteins (G proteins). Dopaminergic D1-like receptors appear to be part of the super-family of G protein–linked receptors. The rhodopsin receptor is the prototype of this super-family, which also includes most receptors for serotonine and noradrenaline. The common features of these receptors are a snake-like appearance (at least, in two dimensions) with seven trans-membrane domains, coiled in the form of spirals with a barrel-like cavity in the centre to receive the ligand, an extracellular N-terminus, and an intracellular C-terminus that can interact with enzymes and the G-proteins. Differently from D1-like receptors, the dopaminergic D2-like receptors have a very long third intracytoplasmic loop. Therefore they are able to interact with G proteins and second messengers while the C-terminus is hypertrophied (Bédard et al. 1995).

The discovery and characterisation of dopamine receptors are largely inter-linked with research on the effects of several antipsychotic drugs. When it was found in 1952, that schizophrenic delusions and hallucinations are blocked by the drug chlorpromazine (a phenothiazine), researchers turned their attention to its sites of action inside the brain. Other antipsychotics belonging to the same or to a different chemical class (like haloperidol, a butyrophenone) were later synthesised and tested. In the 1960´s and 1970´s scientists investigated the target sites where these drugs acted. The search for dopamine receptors was accelerated when a link between certain psychoses and dopamine was established.

Antipsychotic drugs were found to block the dopamine stimulating action of the adenylate cyclase and to affect several steps of the dopaminergic transmission. This enzyme (adanylate cyclase) was associated with a receptor called D1. The response of D1 was the first biochemical response known to be specifically sensitive to dopamine. However, all the blocking actions of the antipsychotics known at that time occurred at much higher concentrations than those found to have clinical relevance. Radiochemical studies using radioactive haloperidol and radioactive dopamine were driven to find out a target in the brain sensitive to the lower antipsychotics concentrations that were effective in clinics. A second, different site of dopamine action was identified. It was called the dopamine D2 receptor. The

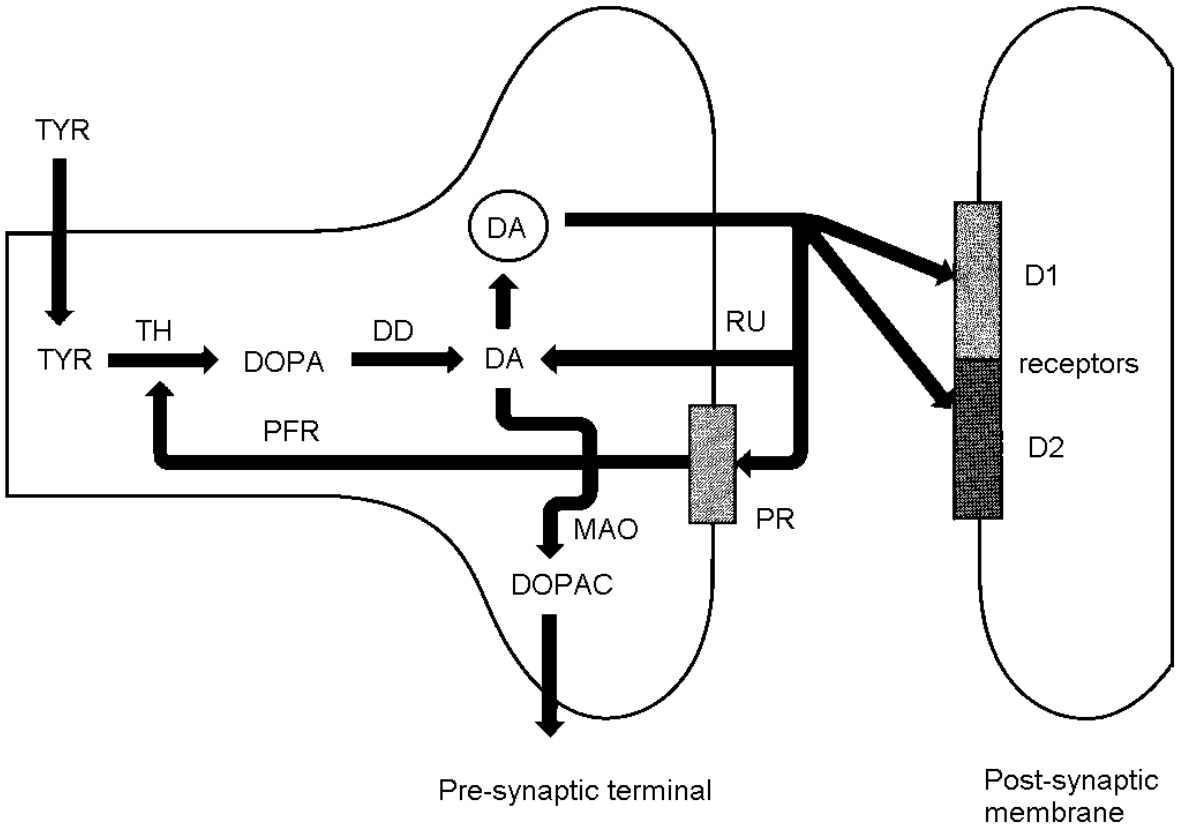


Figure 2.7. The dopaminergic synapse. Tyrosine (TYR) is metabolised to L-dihydroxyphenyl-alanine (L-DOPA) by tyrosine hydroxylase (TH) which in turns is metabolised to dopamine (DA) by dopa-decarboxylase (DD). DA is then stored in synaptic vesicles and released to the synaptic cleft. DA in the synaptic cleft attach either D1-like and D2-like receptors in the post-syantpic membrane and pre-synaptic receptors (PR) in the pre-synaptic terminal where there are also DA re-uptake (RU) mechanisms. The monoamine oxidase (MAO) metabolises DA to L-dihydroxyphenylacetic acid (DOPAC; taken from Riederer et al. 1989).

most influential classification of dopamine receptors was made by Kebabian and Calne (1979) and defines D1 receptors as those linked to the enzyme adenylate cyclase in an excitatory manner, and D2 receptors as those not linked to the adenylate cyclase. A special protein involved in the second messenger pathway of D1-like receptors that will play a role in the first experiment of this thesis is the **d**opamine- and **a**denosine 3',5'-monophosphate-**r**egulated

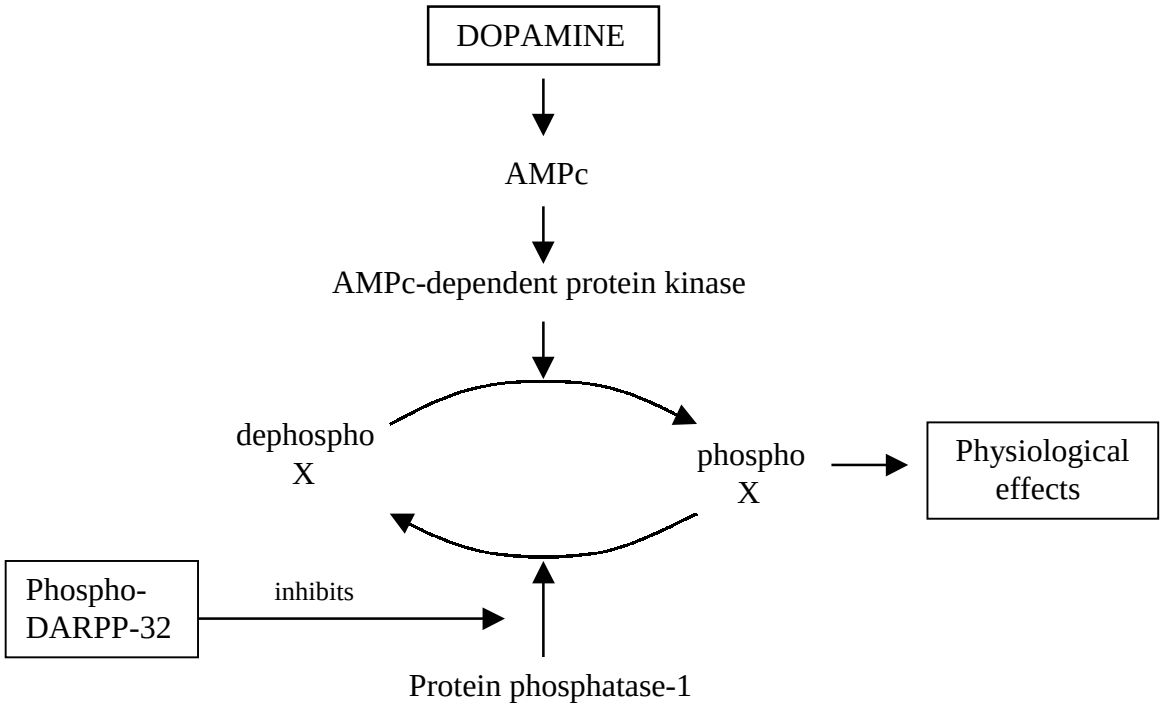


Figure 2.8. Schematic diagram of the positive feedback mechanism by which DARPP-32 is involved in regulating some of the physiological effects of dopamine acting at the D1-like receptor. Dopamine interacting with the D1-like receptor activates adenylate cyclase which increases the intracellular level of AMPc and activates AMPc-dependent protein kinase. These kinase then stimulate the phosphorylation of DARPP-32 and other substrate proteins. Phosphorylate DARPP-32 is an active inhibitor of the protein phosphatase-1. The result of DARPP-32 activity is an enhancement of the AMPc effect (taken from Hemmings et al. 1987).

phospho**p**rotein (DARPP-32). DARPP-32 is phosphorylated by the activity of the dopamine on the D1-like receptor, which induces the adenylate cyclase for the production of **c**yclic **a**denosine **m**ono**p**hosphate (AMPc). The AMPc activates AMPc-dependent protein kinase which stimulates the phosphorylation of DARPP-32 and various other substrates (see figure 2.8). With the development of new dopaminergic drugs and molecular techniques, a total of five different dopamine receptors have been identified: D1, D2, D3, D4, and D5. These receptors differ from each other with respect to their absolute protein sequence and some aspects of their neuroanatomical distribution. However, their pharmacological profiles indicate that they can be grouped into two categories termed D1-like and D2-like. The D1-like includes the receptors D1 and D5 with similar amino acid sequences and the ability to activate the adenylate cyclase, which in turn leads to an increment of the intracellular cAMP levels. The D2-like receptors include D2, D3 and D4 receptors sharing also similar sequences but all inhibiting the adenylate cyclase (Sibley et al. 1993, Andersen et al. 1990). D2-like receptors

may also be linked to other second messenger systems through the activation of K^+ channels, the inhibition of Ca^{2+} channels and/or phosphatidylinositol turnover (Vallar & Meldolesi 1989). More recently, some receptor subtypes within the D1 to D5 receptor types described above, have been identified (Missale & Spano 1996, Waddington et al. 1995). On the pre-synaptic membrane, dopamine autoreceptors, which appear to be mainly of the type D2, have been found (Langer 1997, Chiodo 1992, Goldstein et al. 1990). Here, they mediate a feedback regulation of the dopaminergic transmission. If the concentration of dopamine in the synaptic cleft increases beyond a critical threshold, the autoreceptors are stimulated and induce a reduction in the dopamine synthesis and release (Riederer et al. 1989). For informative reviews about dopamine receptors see Hartman and Civelli (1997) and Palermo-Neto (1997).

Dopamine in the CNS

About thirty years ago, the first efforts to map out the dopaminergic areas of the brain were made. Three main dopaminergic pathways were identified: the nigrostriatal, the mesolimbic and the tubero-infundibular (Ungerstedt 1971, Fuxe et al. 1970, Carlsson et al. 1962). More recently, with the help of more powerful techniques like fluorescence and immunocytochemistry, new dopaminergic systems have been identified and mapped. The most complete and detailed anatomical studies of the dopaminergic system refer to the mammalian brain (Moore & Demarest 1982, Fuxe et al. 1978, reviewed by Fuxe et al. 1985, and dopaminergic systems in primates by Haber et al. 1995). However, among other species, the localisation of different dopamine-containing brain areas in pigeons has been studied and compared with those of mammals (Durstewitz et al. 1999, 1998, Dietl & Palacios 1988, Riechfeld et al. 1987, Reiner et al. 1984). Nevertheless, there are still few studies on dopamine in the avian brain.

In principle, the central dopaminergic neurones can be divided into ascending, descending, and local dopaminergic systems. In the ascending dopaminergic systems, are the nigrostriatal and mesolimbic systems, both involved in the control of motor activity. Muscle tone and body posture are mainly regulated by the nigrostriatal, while movement is controlled by the mesolimbic system. Local injections of dopaminergic antagonists or lesions in the ascending dopaminergic pathways of the mammalian, as well as in equivalent structures of the

avian brain, produce alterations in the motor activity (Fuxe et al. 1985, Staton & Solomon 1984, Goodman et al. 1982).

It was already demonstrated that blocking dopamine receptors with specific dopaminergic antagonists induces rigidity, hypokinesia and tremor, while the action of dopaminergic agonists like L-DOPA leads to the development of involuntary movements, usually of a highly stereotyped character (Delfs & Kelley 1990, Fletcher & Starr 1987). The dopaminergic system not only regulates motor functions but also plays an important role in the control of emotional behaviour (Koch et al. 2000b). The control of the latter is exerted by the mesolimbic system. Several studies have shown its participation in motivation and reward. Neural activity in the mesolimbic dopaminergic system is related to rewarding activities like food ingestion, sexual activity, self stimulation, and drug intake including drug addiction (Martel & Fantino 1996, Richardson & Gratton 1996, Robbins & Everitt 1996, Damsma et al. 1992, Wise & Rompre 1989, Hernandez & Hoebel 1988, Wise 1982). Schultz (1997) reviews the role of dopamine neurones in reward mechanisms in general terms while Diana (1998), Wise (1996a, 1996b) and Di Chiara (1995) review dopamine in human drug-abuse and addiction.

The clinical aspects of the dopaminergic systems are no less important than motor and emotional control aspects, since these two processes are closely related in several diseases. Cellular damage, dopamine imbalance or dopamine receptors alterations of these systems induce for example, Parkinson's disease and Huntington´s chorea, or psychiatric disorders like Schizophrenia and psychosis (Hoes 1998, Koller & Rueda 1998, Brunello et al. 1997, Bédard 1995, Benkert et al. 1995, Carlsson 1995, Seeman 1995, 1987, Riederer et al. 1989).

The descending dopaminergic systems contain neurones originating in the brain and innervating the cervical, thoracic, lumbar, and sacral spinal cord. This dopaminergic system is probably involved in the control of the pre-ganglionic sympathetic neurones activity (Fuxe et al. 1985). There is also a very small dopaminergic system in the spinal cord. This system in the pigeon's spinal cord is described later in this chapter.

Several local dopaminergic systems have been identified in the brain. Some of these seem rather minor and their morphological functionality is not completely understood. Others have been better described and their importance is well recognised. For example, the dopaminergic nerve terminals are also very important in regulating the secretion of several hypothalamic hormones from the pituitary gland, such as the growth hormone, the luteinizing hormone, the

adrenocorticotrophic hormone, and especially the prolactin (Thorner & Vance 1989). These functions are served by the tubero-infundibular pathway, which projects to the hypothalamus and the tubero-hypophyseal pathway, which innervates the hypophysis. Other relevant local dopaminergic systems have been identified in the olfactory and optic areas. It was found that dopaminergic cells located in the olfactory bulb regulate the activity in the olfactory glomeruli via their dendrite processes (Duchamp-Viret & Duchamp 1997, Haláz et al. 1977). The dopaminergic system within the retina is mainly located in the inner nuclear layer and modulates retinal functions via local dendritic projections. Dopaminergic cells have been identified in the retina of several species of amphibia, mammals, and birds, including the domestic pigeon (Glagow & Ewert 1997a, 1997b, Roher & Stell 1995, Djamgoz & Wagner 1992, Schorderet & Nowak 1990).

Pharmacology of the dopaminergic system

In the last four decades, the availability of pharmacological compounds which were relatively less specific at the beginning and more specific later for subtypes of the dopaminergic receptors had progressively increased. There are now several agonists and antagonists to choose from both the D1- and D2-like receptors (Carlsson 1995, Seeman et al. 1993). Considerable progress has been made during the recent. There is already much knowledge about the functions of dopamine-receptors in the brain at the experimental, pre-clinical and clinical levels.

The behavioural stimulation induced by the D2-like receptors at the post-synaptic level is well established, as are the inhibitory functions of the dopaminergic autoreceptors (D2-like receptors). The behavioural stimulation by the D1-like receptors has been extensively demonstrated in animals, but data from clinical studies are scarce (Carlsson 1995). In animals a powerful behavioural interaction between D1- and D2-like receptors is well established. In humans, the fact that L-DOPA is much more powerful than directly acting D2-like receptor agonists in the treatment of Parkinson's disease may be interpreted to indicate a positive D1/D2 interaction, even though the D1-like receptor agonist SKF-38393 has not shown efficacy in this disorder (Braun et al. 1986). Actually, advances in the knowledge of the dopaminergic system and the improvement of biochemical techniques perpetuates the constant design and synthesis

of novel dopaminergic drugs, which in turn allow for better and more specific studies (Arnt 1998, Arnt & Skarsfeldt 1998, Arnt et al. 1997, Reynolds 1997).

Many of the available dopaminergic analogous are not receptor specific, thus they can bind to several dopamine receptor types with different affinities. However, over the last few years much progress has been made in the development of pharmacological agents, which specifically interact with the D1-like or the D2-like receptors, or with one of the five known receptor types grouped under D1- and D2-like. The use of such selective drugs facilitates the study of physiological and behavioural responses associated with different dopamine receptor populations, and may even improve the treatment of disorders associated with certain receptor types.

Apomorphine

Apomorphine was synthesised for the first time in 1869 by Mathiessen and Wright (Lal 1988). It is an alkaloid derivative of morphine, which has no known opioid effect in the organism but acts as a potent dopaminergic agonist (Lal 1988). In figure 2.9 the apomorphine and the dopamine chemical structures are shown together. The dopaminergic action of apomorphine remained totally unnoticed for a long time. This substance was known only because of its emetic properties and indeed, it is still used regularly in clinical practice to induce vomiting. Apomorphine was used in animal experiments which mainly focused on its emetic effects. The animals were often fastened during the experiments, to induce vomiting (mainly in dogs and cats). In order to induce vomiting it is necessary to inject apomorphine at high doses so the observation of any other effect is then impossible. For this reason, the striking ability of apomorphine to induce behavioural stereotypy remained unnoticed for a long time. Harnack, in 1874, interested in the apomorphine effects on animals which cannot naturally vomit, observed some behavioural variations in some species when apomorphine was administrated. Despite of his observations, Harnack and other contemporary researchers could not infer the link to the neuronal system responsible. Apomorphine was recognised as a dopaminergic agonist by Lal (1988) only after dopamine was discovered in 1958.

Apomorphine acts as a direct dopaminergic agonist binding both to the D1-like and D2-like dopamine receptors and it resembles the action of dopamine (Bürki 1984, Colpaert et al. 1976). Apomorphine has pre-synaptic (autoreceptors) as well as post-synaptic effects, its

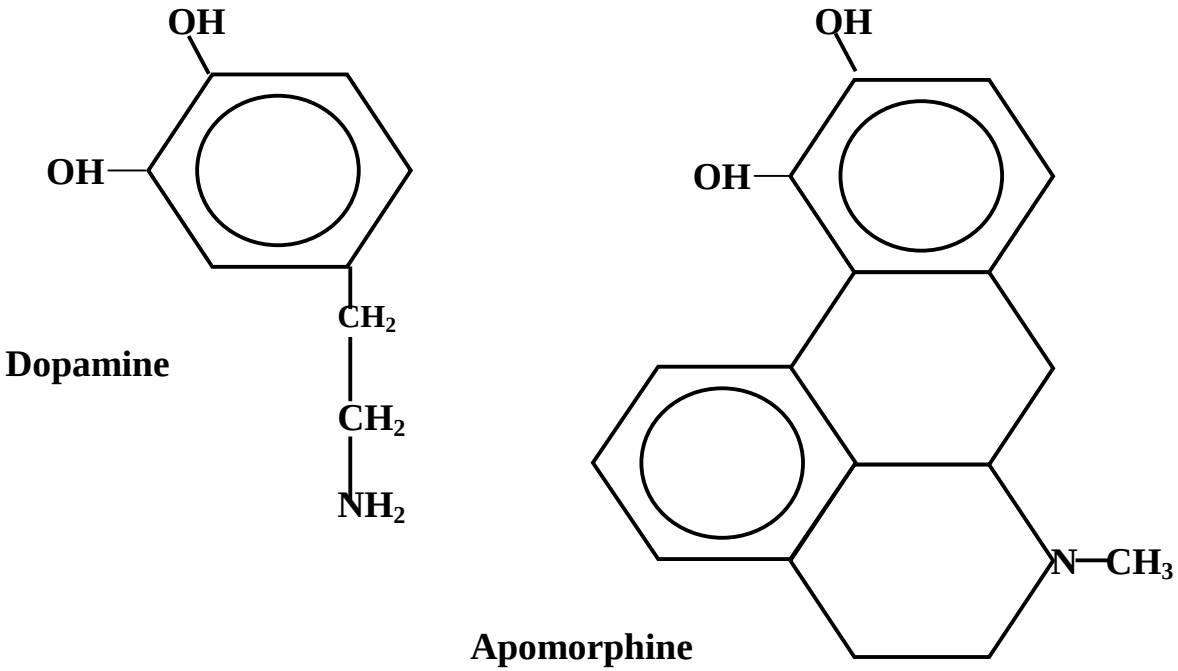


Figure 2.9. The neurotransmitter dopamine and its direct agonist apomorphine (Apo). Dopamine cannot cross the blood-brain barrier while Apo does. Consequently, it is possible to study central dopaminergic functions with relatively less complicated peripheral (e.g. intramuscular) injections of Apo instead of intracranial administrations of dopamine.

action being mainly post-synaptic (Dourish et al. 1989, Lal 1988, German & Arbilla 1984, Schoemaker & Fuchs 1984). Another kind of dopamine agonists are the so called indirect agonists which produce the same effect as the corresponding endogenous substances. Nevertheless, they do not act on the receptors themselves. Indirect agonist, of dopaminergic receptors are for example, amphetamine and cocaine. Amphetamine stimulates the release of dopamine across the pre-synaptic membrane and blocks its re-uptake into the pre-synaptic cell. Cocaine also blocks the dopamine re-uptake (Bürki 1984, Westernik 1979, Moore et al. 1977, Scheel-Kruger 1971). The final result of amphetamine and cocaine is an increase of amounts and/or permanence of dopamine in the synaptic cleft. Antagonists are divided into direct and indirect antagonists. Examples of direct antagonists are butyrophenone, haloperidol and SCH-23390. Haloperidol is a selective D2-like dopaminergic antagonist while SCH-23390 is a selective to D1-like dopaminergic antagonist. Both act directly by binding to

dopaminergic receptors (D1-like and D2-like receptors), keeping the endogenous neurotransmitter from the binding sites and consequently blocking their action (for a better description of these two kinds of drugs see chapters VI and VII).

The importance of the dopamine system in the brain of vertebrates is well known. However, knowledge about the dopaminergic system in the spinal cord, especially of birds is scarce. Avian, as one of the evolution branches of vertebrates, conserved some features of reptilians and some others of mammals. There is seems to be some kind of evolutionary line in which birds appear to be placed between reptiles and mammals in their brain organisation. The next experiment was carried out keeping in mind this basic fact.

Dopamine in the spinal cord of pigeons

The dopaminergic system in a pigeon's spinal cord still remains unclear. At the same time, many aspects of the catecholaminergic system in the spinal cord of vertebrates are not well understood. Consequently, it is of interest to investigate the importance of the dopaminergic system in the pigeon spinal cord to explore a possible involvement of this system on mobility. The purpose of the first experiment of this thesis was to bring some light on the cytoarchitectonic of the dopaminergic system in the pigeon spinal cord. The spinal cord of the pigeon was histologicaly analysed.

Changes in the dopaminergic system as a consequence of phylogenentic evolution are widely accepted. Many studies on cartilaginous and osseous fishes, reptiles and different mammals (Stuesse et al. 1991, Roberts & Meredith 1987) show changes in the dopaminergic systems as a function of the body trunk use (swimming, flying or walking). However, not very much experimental work has been done with birds. Only a few works have been performed in this context because of the absence of a good technique to visualise the system in question. In birds these studies mainly used tyrosine hydroxylase as a marker for catecholaminergic cells (Okado et al. 1991, Chikazawa et al. 1983). Most of the antibodies against dopamine receptors work well in mammals but not so in avian. The novelty of this work lies in the use of a monoclonal antibody against the dopamine neurotransmitter for direct labelling of the dopaminergic cells, in addition to a monoclonal antibody against tyrosine hydroxylase to label the catecholaminergic cells. Both antigens are present in the pre-synaptic membrane. Therefore, only the pre-synaptic cells were labelled. For labelling of the post-synaptic membrane, a second kind of antibody was selected. Antibodies against to DARPP-32 were used to label the post-

synaptic membrane of the dopaminergic synapses. DARPP-32 is a protein associated with D1-like dopamine receptors as described above.

The presence of **t**yrosine **h**ydroxylase (TH) immunoreactive cells and fibres was already demonstrated in the spinal cord of mammals (Mouchet et al. 1986, Dietl et al. 1985), birds (Okado et al. 1991, Chikazawa et al. 1983), lower vertebrates and cartilaginous fishes (Gonzalez et al. 1994, Stuesse et al. 1991, Roberts & Meredith 1987). TH is the first enzyme of the biosynthesis catecholaminergic pathway and catalyses the reaction from L-tyrosine to L-DOPA (figure 2.6).

Catecholaminergic cells and fibres in non-mammal vertebrates are mainly found in two locations. One group consists of neurones contacting the liquor of the central canal, the other involves neurones which do not contact it. The **l**iquor-**c**ontacting **n**eurones (LCNs) are bipolar with an amphora shape and have a single process that protrudes into the central canal (Smeets & González 2000; figure 2.10). Normally, they lie at the ventral rim of the central canal. The non-liquor-contacting neurones have a more diverse location and present differences within different species of non-mammalian vertebrates. For example, in *lampreys*, there are two populations or sub-groups of this neurones. One of them consists of multipolar cell bodies located in the midline region below the central canal, the other consists of few neurones located within the lateral cell column (Pombal et al. 1997, Schotland et al. 1996). In teleosts and birds, non-liquid-contacting neurones were also reported. In the European eel, multipolar dopaminergic neurones were found in the dorsomedial portion of the spinal cord (Roberts et al. 1989) while in the chicken, in the superficial and lateral border of the dorsal horn (Wallace et al. 1996, 1987, Okado et al. 1991). It was proven that dopamine is the most likely neurotransmitter candidate for those catecholaminergic cells. The same is the case for the catecholaminergic LCNs of cartilaginous fish (Stuesse et al. 1991, Roberts & Meredith 1987), bony fish (Roberts et al. 1995, 1989), reptiles (Gonzalez et al. 1994, Smeets & Steinbusch 1990,) and birds (Reiner et al. 1994).

In opposition to the catecholaminergic neurones observed in the spinal cord of non-mammalian vertebrates, the catecholaminergic neurones in the spinal cord of rats are located in the dorsal half part of the grey matter, and do not possess cells with processes contacting the central canal (Mouchet et al. 1986, Dietl et al. 1985). Also, here there is no direct evidence that these catecholaminergic neurones or at least part of them are dopaminergic neurones (Smeets & González 2000).

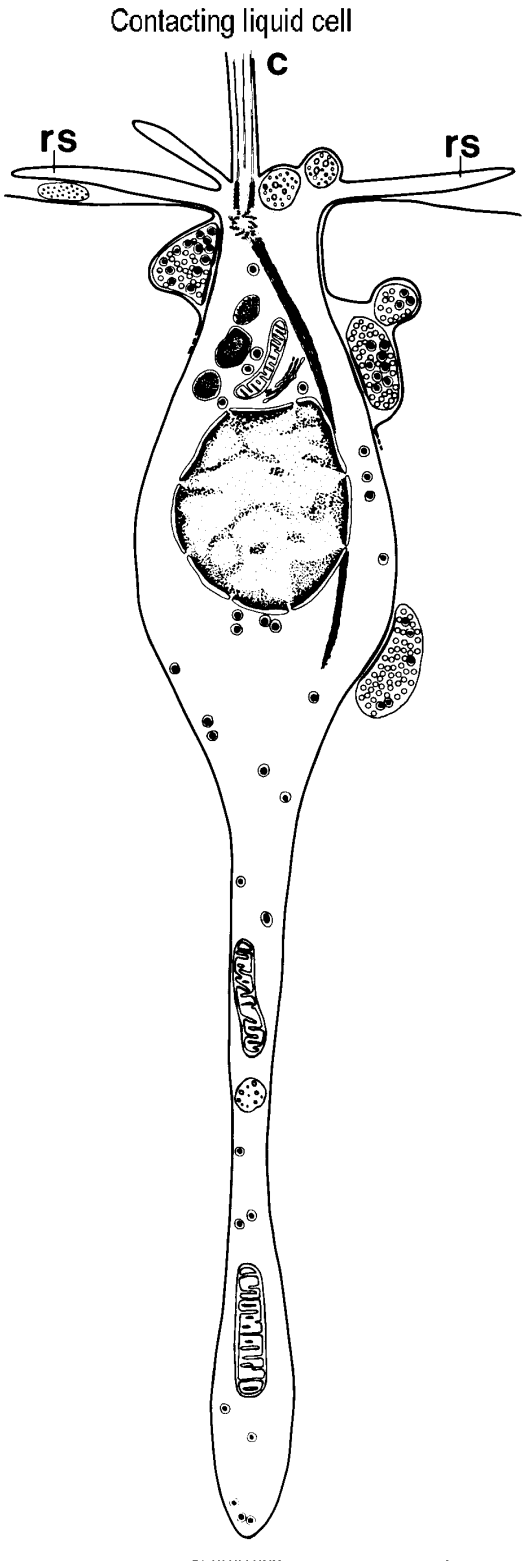


Figure 2. 10 Schematic drawing of the cell body of an LCN. The apical portion of the cell extends as far as the canal lumen into which a cilium (c) and its surrounding rosette (rs) protrude. Ventral to the large nucleus, the basal process extends through the neuropili to pass between the axons of the ventral funiculus (taken from Roberts et al. 1995).

Taken together, it seems that the distribution of the catecholaminergic cells in the spinal cord of cartilaginous and vertebrates species follows a continuum of changes. At one extreme of the scale, catecholaminergic cells are exclusively around the central canal and contact the liquor (Raja radiata, a cartilaginous fish; Roberts & Meredith 1987) at the other rats and monkeys present catecholaminergic cells in the dorsal horn and some others close to the central canal but without contact with the liquor (Uda et al. 1987).

Experiment 1. The aim of this experiment was to study the presence and structure of the possible dopaminergic system in the spinal cord of pigeons, and to compare with the catecholaminergic system of pigeons and others vertebrates. Immunohistochemical studies were performed at different levels of the pigeons' spinal cord. The technique employed here used monoclonal antibodies capable of labelling the pre-synaptic and post-synaptic membranes. Labelling of the pre-synaptic membrane was done by means of antibodies against TH and dopamine. For the post-synaptic membrane an antibody against DARPP-32 was used. DARPP-32 is a protein closely associated with cells expressing the dopamine D1 receptor (Durstewitz et al. 1998, Langley et al. 1997, Schnabel et al. 1997). All immunoreactive-cells for D1 dopamine receptors are also immunoreactive for DARPP-32 (Langley et al. 1997).

Methods

Nine adult pigeons (Columba livia) from local stock were used. Animals were injected with 1,000 IU heparin 15 min prior to perfusion and deeply anaesthetised with 0.35 ml Equitesin per 100 g body weight. The treatment of animals conformed to the specifications of the German law for the prevention of cruelty to animals. For the tyrosine hydroxylase and DARPP-32 immunoreactivity study, six pigeons were perfused intracardially with 300 ml 0.9 % saline (40ºC) followed by 1,000 ml of fixative consisting of 4% paraformaldehyde in 0.12 M phosphate buffer (PB; 4ºC, pH 7.4). After perfusion, spine cords were dissected and stored for one hour in the same fixative to which 30 % (w/v) sucrose was added, and then transferred to 30 % (w/v) sucrose in PB for 12 h at 4º C. For the dopamine immunoreactivity study, the same protocol was repeated with another three pigeons but using 200 ml 0.9 % saline and 2,000 ml of 1 % of paraformaldhyde and 5 % of glutaraldehyde in PB as a fixative, and then in the same fixative with 30 % (w/v) sucrose stored for one hour and then transferred to 30 % (w/v) sucrose in tris buffer (pH 7.2) stored for 12 h at 4º C.

The spinal cords were cut in transversal and sagital slices of 30µm and collected in PB containing 0.05 % (w/v) NaN_3 as a preservative. Spinal cords were transversally cut at level of the cervical nerves: C2, C7, C13; thoraxic nerves: T1, T4; lumbar nerves: L1-L2 and sacral nerves: S1-S2, S6-S7. The sagital cuts were at level of C6, C14 and S3-S5 nerves (Figure 2.11). Slices from every section were prepared for a peroxidase-antiperoxidase (avidin-biotin-conjugate).

Avidin-biotin-peroxidase (ABC) procedure: for ABC technique, slices were treated according to the following procedure: free floating sections were incubated overnight at 4°C in anti-tyrosine hydroxylase monoclonal antibody (anti-TH) from mouse (Boehringer; working dilution 1:200) or anti-DARPP-32 antibody (anti-DARPP-32) from mouse (working dilution 1:10,000) in PBS containing 0.3 % (v/v) triton X-100 (Sigma) pH 7.4; or anti-DA antibody (anti-DA) from rabbit (working dilution 1:500) in tris buffer containing 0.3 % (v/v) triton X-100, pH 7.4. The following steps were carried out at room temperature, separated by three washes of 2 min and two washes of 10 min in PBS (anti-TH and anti-DARPP-32, pH 7,4) or tris buffer (anti-DA, pH 7.6). Slices were pre-incubated in 10 % (w/v) rabbit (for TH and anti-DARPP-32) or goat (for DA) serum in PB or tris buffer respectively. After washing, slices were incubated in the secondary antibody directed against mouse or rabbit from rabbit or goat diluted 1:200 (for anti-TH and anti-DA) or 1:500 (for anti-DARPP-32) in its buffer respectively containing 0.3 % triton X-100 for 1 hour. After washing, slices were put for 1 hour in Vectastain ABC (Vector) in the same buffers as former incubations. For TH and DARPP-32, the normal washes were followed by an additional wash in 0.12 M acetate buffer (pH 6). Staining was achieved by the 3.3' -diaminobenzidine-(DAB) technique with heavy-metal (the addition of heavy metals were only for TH and DARPP-32) amplification by adding $H_8N_2NiO_8S_2$ (2.5 g/100 ml), NH_4Cl and $CoCl_2$ (both 40 mg/100 ml).

For labelling with DAB, 400 mg/100 ml ß-D-glucose were added to the solution. After 10 min of pre-incubation, the reaction was catalysed with 100-200 U/mg glucose-oxydase (Sigma, type VII). In some cases, instead of ß-D-glucose and glucose oxydase, a solution of 0.3% H_2O_2 was used to catalyse the reaction. Finally, slices were washed three more times for 5 min in 0.12 M acetate buffer or tris buffer for DA and two times in PBS.

Results

Results are presented according to the types of labelled cells and fibres: tyrosine hydroxylase (TH), dopamine (DA) and DARPP-32 labelled cells and fibres.

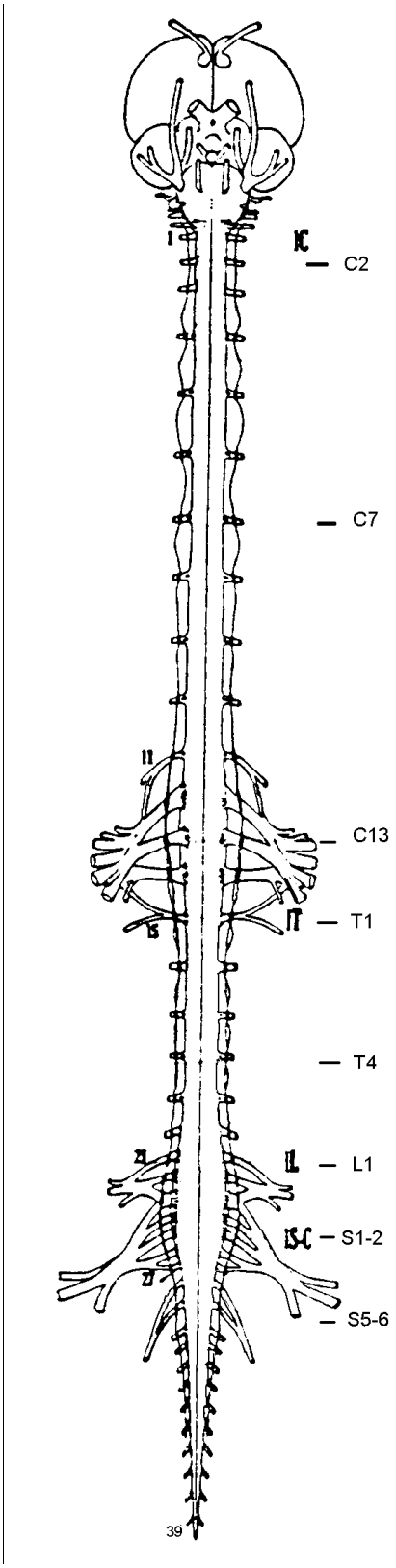


Figure 2.11 Central nervous system, emerging cranial and spinal nerves and sympathetic trunk of the pigeon. There are 39 nerves, 14 cervical, 6 thoracic, 4 lumbar and 15 sacrococcygeal nerves. The letters and number indicate the nerves at which the slices were obtained. C2, cervical 2; C7, cervical 7; C13, cervical 13; T1, thoracic 1, T4, thoracic 4; L1, lumbar 1; S1-2, sacral 1-2; S5-6, sacral 5-6 (taken from Huber 1936).

TH-immunoreactive cells

Mainly two cell populations were found at every transversal and sagital level examined along the pigeons' spinal cords. Figure 2.12a shows a schematic representation of the distribution of TH-immunoreactive cells. The LCNs are located in the laminae VIII (according to Leonard & Cohen 1975; for further details see the lamina in Figure 2.12d) ventrally to the lumen of the central canal. Only a few cells located dorsally to the central canal at sacral level were found. Not all cells lying close to the central canal contacted the central canal liquid. The second population of cells was located mainly in the lamina VI, dorso-lateral to the central canal. In the sagital sections, cells lying ventrally to the central canal were found. They formed a continuos band along most of the spinal cord. The apical portion going to the canal lumen was examined as well as the axon going in ventral and rostral direction from the basal cell portion, as described for non-mammals vertebrates (Roberts et al. 1995). The TH fiber distribution is also plotted in the schematic figure 2.12a. The fibres in the grey matter are present in a reticular fashion while in the white matter, they are in a very clear longitudinal distribution. In the grey matter, they were mainly distributed around the central canal in lamina VIII, in the ventral horn close to the border between the white matter, in laminae VII, VIII and IX, in the lateral part of the dorsal horn in laminae II to VI. In the dorsal part of the lateral columns of the white matter (at every level of the spinal cord) a strong fibre presence running longitudinally was also found.

DA-immunoreactive cells

Along the whole spinal cord two main cell populations were also found. Although these dopaminergic cells seems to be distributed in clusters, they are not so homogeneously distributed as the TH-immunorecative cells are. Figure 2.12b gives a schematic representation of these results. Like the TH-immunoreactive cells, the DA-immunoreactive cells were found in the lamina VIII, contacting the central canal. The cells lying by the central canal are distributed along the spinal cord and have processes to the central canal liquid, as described for the European eel and rainbow trout by Roberts et al. (1995). However, the dopaminergic cells were more distant from each other than TH cells. No cells lying dorsally to the central canal at the sacral level were observed. Also, at the sacral level, cells close to the central canal but without contact with the liquid of the central canal were found. The other group of cells were also present dorso-laterally to the central canal, coincidentally with the TH cells in lamina VI.

Interesting, it was found that some of the TH-immunoreactive cells along the ventral side of the central canal were presumably dopaminergic. In sagital slices a few cells with a regular distribution and equidistant to each other, lying ventrally to the central canal and contacting the

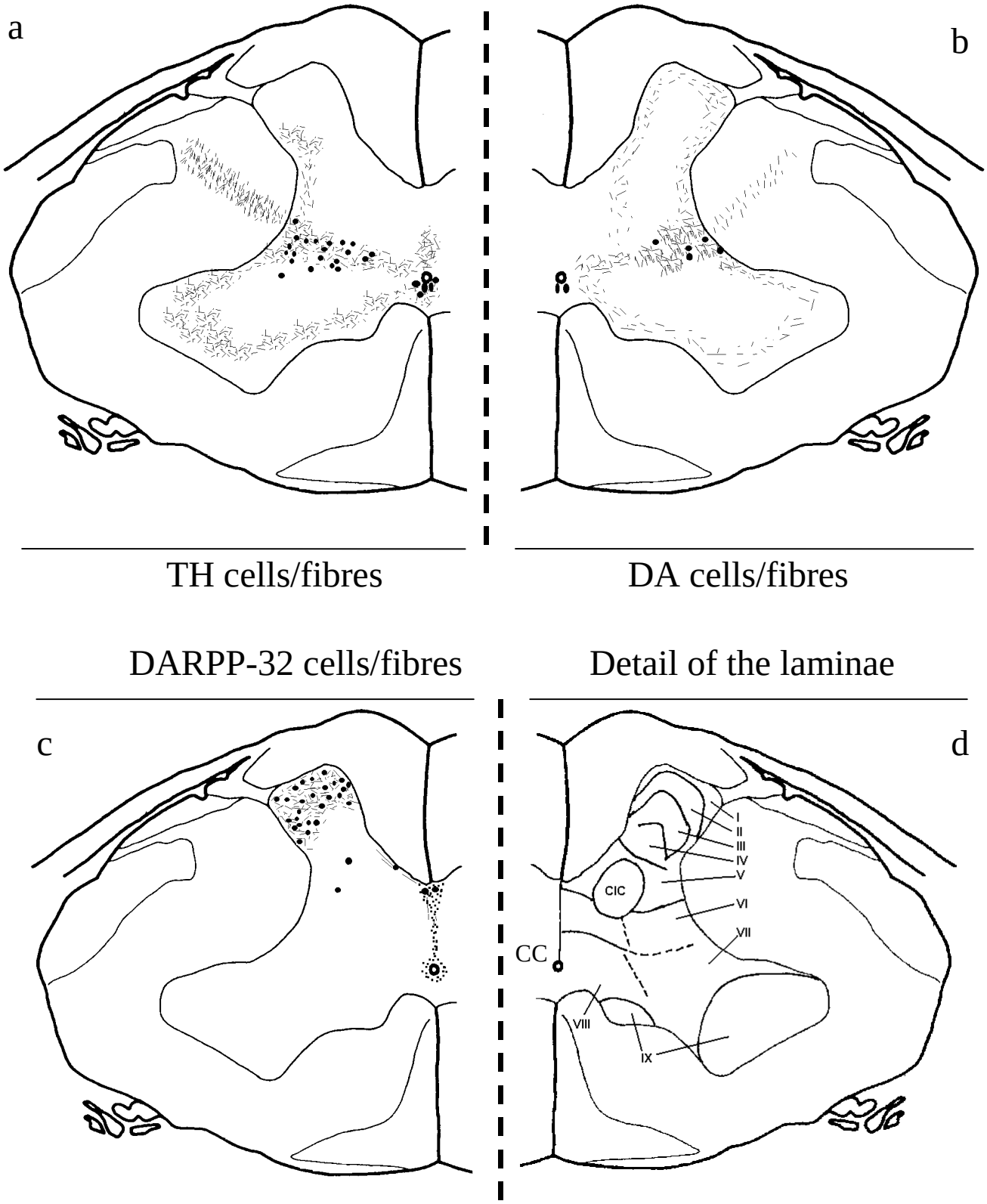


Figure 2.12 Schematic representation of the cell and fibre locations at level of the cervical 12th. Dots symbolising perikarial body cell and small dots and lines symbolising fibres. a) TH-immunoreactive cells and fibres distribution; b) DA-immunoreactive cells and fibres; c) DARPP-32-immunoreactive cells and fibres; d) details of the laminae at level of the cervical 12th. ClC is the Clarke's column and CC is the central canal (according to Leonard & Cohen 1975).

canal lumen were found. The difference compared to the TH-immunoreactive cells was the amount of cells. While TH-immunoreactive cells filled the ventral side of the central canal, the DA-immunoreactive cells were clearly separate from each other.

DA-immunoreactive fibres have a similar distribution to that of the TH-immunoreactive fibres, excepting their distribution around the central canal in lamina VI; here they were absent (figure 2.12b). The rest of the DA-immunoreactive fibres distributed overlapped the TH-immunoreactive fibre patterns. It is also worth mentioning that the number of cells and fibres labelled using anti-DA was lower when compared with the slices labelled with anti-TH antibody.

DARPP-32-immunoreactive cells

The post-synaptic membrane of the synapses, containing the D1 dopamine receptor (labelled with anti-DARPP-32) showed three clear groups of cells (Figure 2.12c). The most popular group was located in the dorsal horn in laminae I, II and III. A second group was located also in the dorsal horn but more ventrally, in the bases of the horn in lamina IV and V. These cells were present in almost every slice but only one or two per slice, making a line of cells along the spinal cord. The third group was found in lamina V but lying at the rim of the grey matter. Most of the cells belonging to the third group were located centrally, at the rim of the grey matter. However, some of these cells lay at the rim but close to lamina IV, in the dorsal horn. Apparently, a fourth group of cells was found more caudal in the spinal cord, at the sacral level. They were in the lamina VI exactly behind the rim cells from the third group. This fourth group seems to be part of the rim cells. If not it should be very closely related because of the location and also because they only appear at the sacral level as a branch of the rim cell population. This fourth group seems, therefore, to be a specialisation of the dopaminergic system.

The distribution of the DARPP-32 fibres clearly forms three groups. One of them is very abundant in the dorsal horn and overlaps the cell distribution in laminae I, II and III (Figure2.12c). These fibres present a reticular shape. A second group is of special interest because of its distribution around and very close and dorsally to the central canal (where the presence of dopaminergic cells is important), and over the central line, in between the central canal and the rim cells group. Also, strongly labelled fibres from the rim cells were found. This third group of fibres runs parallel to the grey matter border toward the dorsal horn. They also seem to have their origin in the rim cells.

Discussion

The existence of the diencephalo-spinal dopaminergic system has been well-demonstrated by both biochemical and anatomical studies. These studies were carried out mainly in mammals. This system has its origin in the dorsal medial hypothalamus-caudal thalamus group (A11 area in the rat) and projects mainly to zones around the pre-ganglionic sympathetic neurones in the spinal cord at the level of the thoracic-lumbar nerves (Maisky & Doroshenko 1991, Lindvall et al. 1983). However, it was also demonstrated that there are dopamine body cells containing both dopamine D1- and D2-like receptors in the spinal cord of rats (Van Dijken et al. 1996, Dubois et al. 1986). These findings support the hypothesis of Commissiong and Neff (1979) proposing the existence of an independent intrinsic dopamine system apart of the descending fibres from area A11. Previous studies demonstrate that the administration of L-DOPA in the spinal cord of rats and cats results in potent and specific effects on neuronal elements associated with the mono-synaptic reflex, the tonic stretch reflex and the flexor reflex in these animals (Geber & Dupelj 1977, Nygren & Olson 1976, Grillner 1969). The effect of L-DOPA on the mono-synaptic reflex in the spinal cord was specifically blocked by pimozide (Geber & Dupelj 1977), a D2-like dopamine receptor antagonist. It was demonstrated later that the mono-synaptic mass reflex was depressed by apomorphine and furthermore that this depression needed the activity of D1- and D2-like receptors (Gajendiran et al 1996). Other research work shows that the blunted pressor response to quinpirole is reverted by a spinal depressor effect mediated by a dopamine receptor (Lahlou 2000). All these experiments were performed on animals which were cord-transected at the level of the first cervical segment or decerebrated in some other way. Commissiong & Neff (1979) also hypothesised that the effect of L-DOPA is exerted on dopaminergic terminals and not on the norepinephrine terminals. This hypothesis was then supported by experimental evidence (Commissiong et al. 1984). The norepinephrine terminals are more related to the descending catecholaminergic fibres of the coerulo-spinal projection and other brain nuclei (Commissiong et al. 1984).

On the basis of this anatomical and functional background, the previous results obtained from immunocytochemical studies performed in the pigeons' spinal cord will be now reviewed.

Pre-synaptic membrane

The immunocytochemical studies demonstrates that most of the catecholaminergic cells and fibres labelled with anti-TH are co-located with the dopaminergic cells labelled with anti-DA. DA- and TH-immunoreactive cells were located along the spinal cord ventrally to the central

canal showing regional aggregation. For example, they were very scarcely distributed in the medial cervical region (C6-C7) and more abundant in the rostral cervical (C2-C3) and sacral (S5-S6) regions. In the rest of the levels studied (thoracic and lumbar) the presence of the DA- and TH-immunoreactive cells were intermediate.

The amount of DA-immunoreactive cells and fibres was lower than the amount of TH-immunoreactive cells and fibres respectively. To explain this difference two possible reasons could be argued. First, there are fewer DA-immunoreactive cells and fibres than TH-immunoreactive cells and fibres. Some TH-immunoreactive cells and fibres could be used as epinephrine or norepinephrine neurotransmitter. For example, some studies demonstrate that norepinephrine is about ten times more abundant than dopamine cells in the spinal cord of rats (Commissiong & Neff 1979, Commissiong et al. 1978). Second, some differences in the technique that use anti-TH and anti-DA could be responsible. These differences could be related more or less to the anti-DA monoclonal antibody. For example, the anti-DA could not label with the same efficacy the dopaminergic cells and fibres that the anti-TH did with the catecholaminergic cells and fibres. Considering the antigen used, DA is a soluble substance which can be washed very easily from cells during the different steps in the immunocytochemistry technique despite the special care given to avoid it. Nevertheless, to test the efficacy of the anti-DA antibody, parallel to the spinal cord, the same immunocytochemistry technique was also carried out with brain preparations. These showed a very clear and strong labelling of dopamine cells in the substantia nigra and other mesencephalon areas, as for example in the ventral tegmental area. A profuse distribution of dopamine fibres was also found in the whole striatum. The presence of the terminals of the nigrostriatal and mesolimbic dopaminergic systems as well as other typical dopamine structures in the pigeon brain is well known. Consequently, it seems to be the case that the few dopaminergic cells and fibres labelled approximately corresponds to the same proportion of catecholaminergic cells and fibres described for other species.

While the distribution pattern of TH immunoreactive cells partially agrees with previous data from chickens, the DA immunoreactive cells in pigeons do not have the same location. Wallace et al. (1987) found in embryo and hatching chickens two conspicuous TH-immunoreactive cells populations: cells situated ventral to the central canal and cells mainly situated in the lateral border of the dorsal horn, from bases to top (layers I, II, V and VI; according to Martin 1979). Only cells situated in layer VI of chicks are close to the TH-immunoreactive cells of pigeons situated in layers V and VI. However, in the dorsal horn of the pigeon neither TH-immunoreactive cells, nor DA-immunoreactive cells were found. Wallace et

al. (1996) found only very few DA-immunoreactive cells along the whole spinal cord of the hatching chicks. These cells were always situated ventral to the central canal. Even in hatching chickens treated with L-DOPA and MAO inhibitors (to increase the intensity of the stain of the DA-immunoreactive cells), only DA-immunoreactive cells ventral to the central canal were found.

Previous studies indicate that the LCNs in the paraventricular organ (in avian this organ forms a bilateral small groove on the third ventricle wall) of non-mammalian vertebrates accumulates rather than synthesises dopamine, since these cells in chicks as well as amphibians exhibit DA- and not TH-immunoreactive cells (Smeets & Gonzales 1990). Similar results were found in lizards (Smeets et al. 1991). The authors conclude that these cells accumulate dopamine and that the cerebrospinal fluid plays an important role in dopamine neurotransmission in non-mammalian vertebrates. Other studies supporting this hypothesis were done by von Bartheld and Meyer (1990). They injected intra-ocularly cobaltous lysine in the lungfish. This resulted in a selective silver impregnation of the neurones in the paraventricular organ. They also conclude that this impregnation is the result of the uptake of the tracer from the vascular circulation. In pigeons no double immunocytochemistry study was carried out to determinate if the DA containing cells also contain TH. Also, with the studies presented here, it is not possible to test the above mentioned fact. Nevertheless, the amount of TH-immunoreactive LCNs formed a complete band along the central canal of the spinal cord. Therefore the DA-immunoreactive LCNs could be included among them. These contrasting results could be explained by taking into account the fact that these two dopaminergic LCNs populations are functionally different. Guglielmone (1995) compared the LCNs containing DA from the paraventricular organ and those from the spinal cord of quail embryos. He concluded that these cells represent two different subsets of LCNs. The respective functional roles of these cells still remain to be investigated.

The second cell population located more laterally in the VI lamina could play an inter-neuronal role. This is because of the great evidence that dopamine may mediate motor activity. It was demonstrated that the dopamine agonist, apomorphine, and dopamine metabolite, L-DOPA, mediates the flexor reflexes of rats and mass reflexes of cats respectively (Geber & Dupelj 1977, Nygren & Olson 1976). However, the descending pathway from the hypothalamus (A11 cells in the rat) to the spinal cord has its main dopaminergic projection on the sympathetic inter-mediolateral cell column and sympathetic pre-ganglionic neurones (Gladwell & Coote 1999a). It is not clear which is the target of the dopamine neurones in laminae VI. However, some speculation about functionality according to cell locations can be

done. From this point of view, a more conservative interpretation could be that such neurones project to the motoneurones in the ventral horn. Nevertheless, until now there is no evidence that these cells play an inter-neuronal role. Consequently, other experiments are needed to ascertain the exact functionality of such dopaminergic neurones in the pigeon spinal cord.

Post-synaptic membrane

The existence of DARPP-32-immunoreactive cells in the spinal cord of pigeon is a completely new finding despite the fact that there are not many studies performed with anti-DARPP-32 antibodies. The presence of the dopamine D1-like receptors was verified in the whole spinal cord of pigeons. DA has a great influence in many of the motoric functions of the spinal cord. However, dorsal horns and not ventral ones, had so many DARPP-32-immunoreactive cells and fibres. The ventral horns are more related to motoric functions while the dorsal ones are related to sensorial input. In this context, it is valuable to point out that DARPP-32-immunoreactive cells only serve as a marker for dopamine D1- but not for dopamine D2-like receptors. Therefore, only D1-like-immunoreactive cell populations are labelled. The dopamine D2-like population still remains to be studied. In the rat spinal cord both cell populations, D1- and D2-like are present. They differ, however, in the sub-regional localisation (Dubois et al. 1986) and have also clearly different functions (Hasegawa et al. 1990). This independence is not true for every aspect of the dopaminergic functions in the spinal cord. It was demonstrated that some dopamine D1- and D2-like receptors are functionally coupled in the rat spinal cord (Gajendiran et al. 1996).

The question whether DARPP-32 neurones receive input either from dopaminergic neurones in the spinal cord or as input from the diencephalo-spinal system is still not answered. It is possible that the DARPP-32-immunoreactive cells from the dorsal horn receive projections from the dorsal root ganglion. Despite the lack of evidence that these ganglion cells contain dopamine, the localisation and number of the DARPP-32-immunoreactive cells in the dorsal horn could indicate that they receive their sensorial input from the dorsal root ganglion. Of course this remains speculative and further experiments are needed to clarify this issue.

The DARPP-32-immunoreactive cells located in lamina V at the basis of the dorsal horn and at the rim of the grey matter could serve as inter-neurones. Not many of these cells were found. The amount corresponds more or less to the amount of DA-immunoreactive cells, which lay in laminae V and VI. There are around the central canal profuse dopaminergic fibres in laminae V-VI as well as in lamina VIII. These DARPP-32-immunoreactive cells could be modulated somehow by dopaminergic cells from the spinal cord and/or supra-spinal

projections. In so doing the sympathetic reflex involving the DARPP-32-immunoreactive cells may be modulated by intra-spinal dopamine activity. There is some evidence that dopamine modulates the sympathetic reflex (Horn & Kohli 1992, Lewis & Coote 1990). However, these authors could not confirm whether these reflexes were mono- or poly-synaptic. Therefore, it could be hypothesised that the sympathetic reflexes modulated by dopamine have an intra-spinal dopamine input. Retrograde labelling of the dopamine D1-like receptors of sympatho-adrenal pre-ganglionic neurones in the spinal cord of rat showed that the D1-like receptor localisation on target-specific neurones was present in more than half of the spinal cord sections studied (Gladwell et al. 1999). The localisation was also associated with the cell soma and principal proximal dendrites in the inter-mediolateral cell column of the spinal grey matter (Gladwell et al. 1999). Additionally, Gladwell and Coote (1999a, 1999b) reported that dopamine may exert an indirect excitatory influence on sympathetic pre-ganglionic neurones, possibly via inter-neurones present in the spinal cord.

Dopaminergic functionality in the pigeon' spinal cord

Low doses of dopamine into the lampreys' spinal cord cause a rate acceleration of fictive swimming. This is induced by glutamate. Nevertheless, at higher concentrations it slows this rhythm (McPherson & Kemnitz 1994). In pigeons, dopamine seems to play a role in visceral functions more than to modulate the locomotor activity. The finding of both dopaminergic cell populations together with the location of the DARPP-32-immunoreactive cells, suggests that dopamine could innervate neurones of the dorsal horn as well as neurones at the rim of the grey matter. Keeping in mind the cluster distribution of the DA- and DARPP-32-immunoreactive cells, it could be thought that dopamine has some effect at the cervical and sacral levels. At these levels they are in elevated number. Neck and pelvic regions could be controlled by dopaminergic activity in these regions.

Until now, every investigation about catecholaminergic cells in the spinal cord of birds was carried out in hatching and embryo birds. In this thesis results about dopaminergic cells in adults birds are presented. It has been already demonstrated in rats, that the early gene expression of dopaminergic D1- and D2-like receptors in different brain areas implies that receptor expression is an intrinsic property of these neurones. Additionally, the expression of the D1-like receptor in the spinal cord of rats was observed on the gestational day 14, while the expression of the D2-like receptor appears at birth (Schambra et al. 1994). A lot of further research must be done on the dopaminergic system in the spinal cord of birds as well as of other vertebrates.

The main objective proposed before examining the dopaminergic system in the pigeon' spinal cord was achieved. This work contributes to understanding the dopamine system in the spinal cord of birds as well as their catecholaminergic system. It is extremely important understand the activity of dopamine in the whole animal body, especially when dopaminergic drugs are used for behavioural experiments. The next chapter describes a classical conditioning learning paradigm. Here, the dopaminergic agonist apomorphine was used as an unconditioned stimulus.

Chapter III

Conditioning induced by apomorphine

Introduction

As explained in the general introduction, associative learning involves the development of a connection between the internal representation of two events, for instance two stimuli or a stimulus and a response, so that the occurrence of one activates the representation of the other (see Domjan 1993). In the experiments presented in this and the following chapters, two different associative learning paradigms are used to assess the role of the dopaminergic and glutamatergic neurotransmitter systems in the learning processes. One of these paradigms is the previously mentioned apomorphine-induced classical conditioning, described below, while the other is a novel drug-free simultaneous discrimination paradigm, which will be described in chapter IV.

Classical conditioning, first described by the Russian psychologist Ivan P. Pavlov in 1927, is a form of associative learning dependent upon the co-occurrence of two stimulus events. In classical conditioning, a certain stimulus, the so called unconditioned stimulus (US) elicits a spontaneous biological response. This response is normally highly reflexive and is called the unconditioned response (UR). A second stimulus, the so called conditioned stimulus (CS) does not elicit that response at all. After repeated, consistently paired presentations of the US and the CS, the latter eventually comes to elicit a conditioned response (CR), which is normally similar to the UR. The CS can be a discrete stimulus such as a flash, a tone, or a figure, or it can consist of contextual cues such as visual features of the experimental situation, the manipulation, or the time of the day in which the experiment is carried out. When the contextual cues serve as a CS, the classical conditioning situation is called “context conditioning”. The US is such a stimulus that invariably elicits a conditional response in the animal. Typical USs are food delivery to hungry animals, mild electrical shocks, air puff to the eyes and, in principle, any stimulus that produces a reliable unconditional response. Therefore, the effects of certain drugs, which reliably induce a particular response in the organism, may be used as suitable USs. This is called drug-induced classical conditioning.

The classical conditioning mediated by the direct dopaminergic agonist apomorphine mentioned in the introduction, consists of the association between the effects that

apomorphine produces in the subject (US) and the contextual cues, in other words where the subject experiences those effects (CS). Apomorphine-induced conditioning will be described in some detail in this chapter. Additionally, new evidence about the long-lasting retention of the association between apomorphine effects and contextual cues will be presented, and the possible influence of such a learning process on the numbers and affinity of the dopamine receptors in the pigeon's brain will be assessed.

Apomorphine induces stereotyped activity

Apomorphine exerts noticeable effects on the behavioural activity of several species, inducing motor activity and often leading to the development of stereotypy. Stereotypic behaviour refers to actions that occur repetitively with very little variation and which have apparently no goal or function for the organism (Ridley & Baker 1982). Amsler (1923) reported for the first time that in rats and guinea pigs, administration of apomorphine produced stereotyped repetitive chewing. Since then, many authors have reported several different stereotypic behaviours induced by apomorphine in diverse animal species, for example, snails, toads, fishes, tortoises, mice, rats and humans (Glagow & Ewert 1997a, 1997b, Szechtman et al. 1987, Ljungberg & Ungerstedt 1977, Andersen et al. 1975, Fekete et al. 1970). In all the species studied, apomorphine induces augmentation of the respiration rate and increases general motility (Lal 1998, Harnack 1874). In rats, apomorphine induces intense repetitive sniffing, licking and chewing, and increased reactivity to environmental stimuli (Essman et al. 1995), as well as hyperactivity during running and rearing (Havemann et al. 1986). Additionally, apomorphine has been found to increase climbing in mice (Cabib & Puglisi-Allegra 1988, Kendler & Davis 1984) and guinea pigs (Frommel et al. 1965), sniffing in cats (Motles et al. 1989) and rabbits (Hill & Horita 1972, Harnack 1874) and chewing in dogs (Nymark 1972), monkeys (Shintomi & Yamamura 1975) and humans (Lal 1998). It also induces increased leg and wing movements and head shaking in pigeons (Lindenblatt & Delius 1987, Goodman 1981, Basten-Kreft 1977). As it can be noticed, apomorphine frequently induces oral stereotyped movements, like chewing or pecking. However, if the administered apomorphine doses are high, they induce vomiting in almost all species, except those lacking the biological disposition to vomit, for example fish (Tiersch & Griffith 1988). Specifically in pigeons and chickens, low apomorphine doses induce bouts of repetitive pecking, a response identified and described many years ago (Dhawan et al. 1961, Dhawan & Saxena 1960). This consistent stereotyped pecking response has been extensively used in

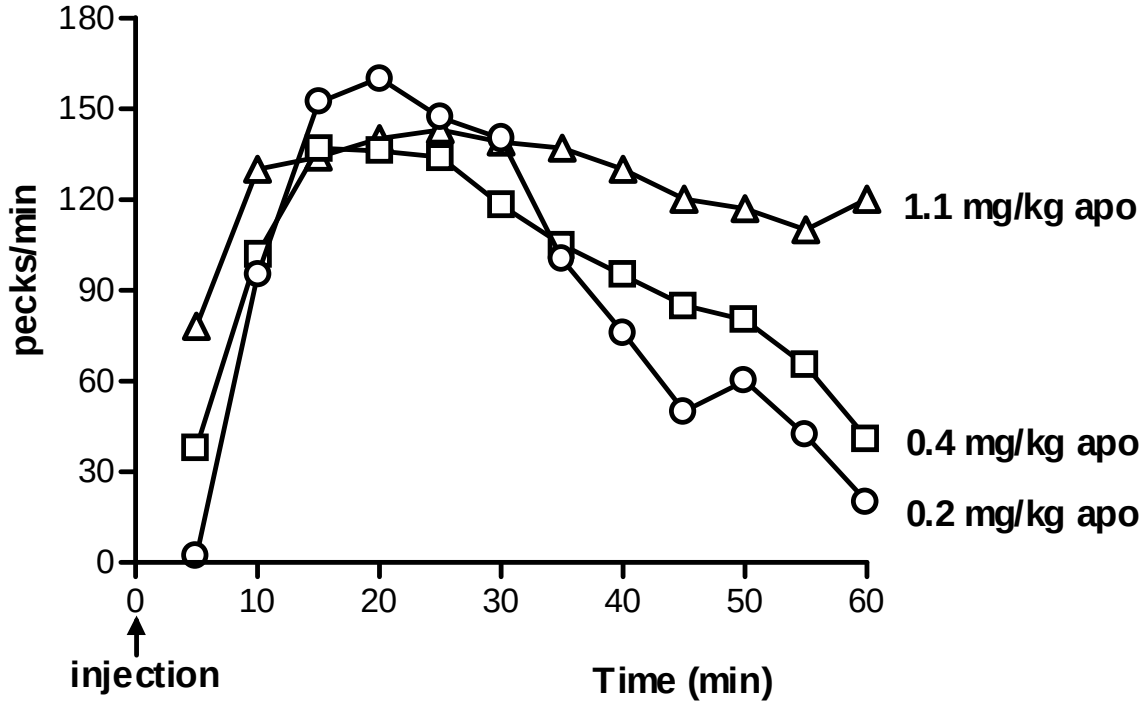


Figure 3.1. Time course of the pecking response (n = 4) during the 60 minutes after a single apomorphine injection of 0.2, 0.4 or 1.1 mg/kg. For the three apomorphine doses, the response increases and reaches a maximum about 20 minutes after injection (taken from Basten-Kreft 1977).

behavioural experiments to study the role of dopamine in behaviour and learning. Additionally, it has provided a reliable test for the efficacy and potency of different dopaminergic drugs (Akbas et al. 1984, Cheng et al. 1975, Cheng & Long 1974).

As mentioned, an intramuscular (i.m.) injection of a relatively low apomorphine dose (about 0.1 and 2.0 mg/kg) into pigeons induces a bout of repetitive pecking. After injection, the response begins within a few minutes, increases to a maximum value, about 20 minutes later, then starts to decrease. The total reaction lasts between one and one and a half hours and consists of several thousand pecks (Machlis 1980, Brunelli et al. 1975). Figure 3.1 exemplifies the time course of the pecking response after apomorphine injections of 0.2, 0.4, or 1.1 mg/kg from an experiment by Basten-Kreft (1977) in our laboratory. The apomorphine doses used in behavioural experiments with pigeons usually range between 0.1 and 2 mg/kg. Doses higher than 2 mg/kg have been found to induce co-ordination impairments, which interfere with the pecking response (Basten-Kreft 1977). Apomorphine-induced pecks can be directed to food grains, however, they normally do not lead to grain ingestion. Actually, apomorphine has been found to have anorexic effects in pigeons (Deviche 1984).

Nevertheless, the movement patterns of the apomorphine-induced pecks are very similar to those of foraging pecks (Siemann & Delius 1992a). Of the other dopaminergic agonists, only amphetamine has been found to elicit weak pecking, although within a narrower dose range, with lower potency and higher toxicity (e.g. Kraemer et al. 1997, Schaal et al. 1995, Goodman 1981, Cheng et al. 1975). While pigeons injected with an effective apomorphine dose display a high degree of repetitive pecking, control pigeons injected with a saline solution (Sal) in the same conditions will occasionally peck only a few times (see figure 3.2).

Apomorphine induces behavioural sensitisation

When a certain effective apomorphine dose is repeatedly injected into pigeons, the total number of pecks elicited by that dose progressively increases with each injection, until it reaches a dose-dependent asymptotic level. Such an increment in the efficacy of a given drug-dose as a consequence of a prolonged treatment is referred to as drug sensitisation. Pecking sensitisation in pigeons has been observed over a dose range of 0.1 to 2.0 mg/kg apomorphine (Brunelli et al. 1975). Equivalent apomorphine-induced behavioural sensitisation has been found for other responses in mice, rats and humans (Mattingly & Gotsick 1989, Szechtman et al. 1987, Carey 1986).

The pecking sensitisation of pigeons is a very well known, consistent and reliable phenomenon, which has been replicated many times for several different apomorphine doses, ranging between 0.1 and 2.0 mg/kg, in our laboratory and elsewhere (e.g. Godoy & Delius 1999, Wynne & Delius 1995, Brunelli et al. 1975). Figure 3.2. shows an example which illustrates typical sensitisation curves obtained with a relatively small, a medium and a relatively high apomorphine dose (0.2, 0.5 and 1.0 mg/kg respectively). As can be observed, the shape of all the three curves that reflect the pecking sensitisation do not seem to vary significantly among doses. However, the asymptote is clearly dose-dependent.

There are several other examples of sensitisation caused by repeated administration of drugs such as amphetamine, cocaine and apomorphine, on different behavioural responses in different species. The two first psychostimulants, as explained before, are rather unspecific, indirect agonists of dopamine, which act mainly pre-synaptically (Nicola et al. 1996, German & Arbilla 1984) while apomorphine is a potent specific and direct dopaminergic agonist, which acts mainly post-synaptically (Kruk & Pycock 1991, Schoemaker & Fuchs 1984). Many of the motor activating and stereotypic inducing effects, as well as the psychostimulatory effects induced by these drugs, sensitise upon repeated administrations of a certain dose (Kuczenski et al. 1997, Mattingly et al. 1997, Anagnostaras & Robinson 1996,

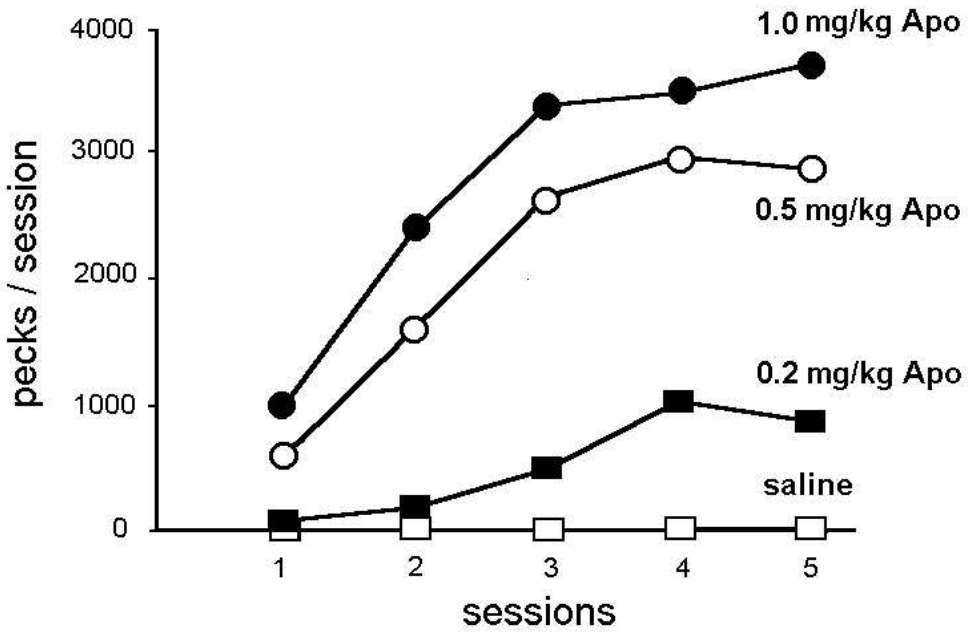


Figure 3.2. Apomorphine-induced pecking sensitisation with three different doses: 1.0 mg/kg, 0.5 mg/kg and 0.2 mg/kg apomorphine, as compared with the pecking response of saline injected pigeons. A clear dose-response asymptotic relationship was observed (taken from Godoy 2000).

Burechailo & Martin-Iverson 1996, Mattingly & Gotsick 1989, Carey 1986). The mechanism underlying the dopaminergic drug-induced sensitization is however, a much disputed issue (Anagnostaras & Robinson 1996, Bedingfield et al. 1996, Kuribara & Uchihashi 1993, Stewart & Vezina 1988, 1991).

The mechanisms of apomorphine-induced pecking sensitisation

The simplest, most intuitive explanation for apomorphine-induced pecking sensitisation would be the physical accumulation of the drug inside the organism, as a result of repeated administrations, which would cause the progressive increment in response magnitude (Kalant 1989). In that case, longer inter-injection intervals would yield less sensitisation than shorter ones. However, results obtained in our laboratory demonstrate that lengthening the inter-injection interval from one to three days increases the magnitude of the sensitisation instead of reducing it (Keller, personal communication), which weakens of the possibility that sensitisation results from drug accumulation.

Two other alternative mechanisms have been proposed to underlie drug sensitisation namely, straight pharmacological and/or biochemical processes or conditioning processes.

Based on these possible mechanisms, in our laboratory two different hypotheses about the causes of apomorphine-induced pecking sensitisation in pigeons have been proposed. The first hypothesis, called "the pharmacological hypothesis", assumes that the sensitisation is the outcome of a purely pharmacological process. It can be postulated, for example, that apomorphine produces a change in the efficacy of the relevant synaptic transmission by modifying the number of dopamine receptors or altering their affinity, so that the response to subsequent apomorphine administrations would be different, i.e. progressively more intense, until a maximum is reached. The second hypothesis, called the "conditioning hypothesis" assumes that apomorphine initiates learning processes that would indirectly modify the effectiveness of the relevant synapses. Several experimental results support the idea that apomorphine is involved in learning processes and that learning is responsible for, at least a part of the pecking sensitisation of pigeons. However, it should be kept in mind that there is no reason to assume that both types of mechanisms must be mutually exclusive. Actually, many authors think that the response sensitisation induced by cocaine and amphetamine in rodents is due to a combined pharmacological-learning process (Lienau & Kuschinsky 1997, Burechailo & Martin-Iverson 1996, Crombagh et al. 1996, Stewart & Badiani 1993, Kalant 1989).

Many experimental results strongly suggest that the apomorphine-induced pecking sensitisation is due to classical conditioning. Apomorphine induces repetitive pecking even in absolute darkness (Leydel 1999) but normally, visual environmental cues modulate the pecking response (Keller & Delius 2001). Pecks tend to be directed at small visually contrasting stimuli present in the experimental context. Such stimuli also seem to increase the frequency of pecking. If no visual contrasting features are available, pigeons tend to peck at the cross-welds of their steel grid home cages, dust-specks or even on their own claws (Basten-Kreft 1977, Brunelli et at. 1975). Lindenblatt and Delius (1987) demonstrated that the apomorphine-elicited pecking response can be classically conditioned to visually distinctive contextual cues by using a Pavlovian differentiation design. They repeatedly injected a group of pigeons with an effective apomorphine dose in a distinctive experimental cage, whose walls were lined with white cardboard peppered with green dots. The same animals were injected Sal in a different experimental cage, whose walls were lined with black cardboard peppered with yellow dots. During the subsequent test, only with Sal injections, pigeons pecked more in the first cage than in the second. It was concluded that apomorphine acted as unconditioned stimulus (US) eliciting an unconditioned pecking response (UR) and that the visual cues been present in the first experimental cage acted as conditioned stimuli (CS).

After a training with repeated apomorphine injections in that cage (equivalent to repeated paired US-CS presentations), the pigeons exhibited repeated conditioned pecking (CR) in response to that environment even without apomorphine. Naturally, in order to control for possible colour preferences a second group of pigeons was trained with apomorphine in the black/yellow cage and with Sal in the white/green cage. Nevertheless, no such preferences, were found. The fact that these results were obtained using a Pavlovian differentiation paradigm makes possible artefacts caused by the novelty/familiarity of the test procedure and context rather improbable.

Burg et al. (1989), using a place conditioning design, demonstrated that pigeons, treated with apomorphine in a certain cage and with Sal in another one, consistently preferred to enter the former rather than the latter, when given the option during a subsequent test with Sal injections. It is a frequent finding that, as a classical conditioning training progresses, the time interval between the presentation of the US and the onset of the response (CR/UR), i.e. the latency, progressively shortens (Domjan 1993). Wynne and Delius (1995) trained pigeons with repeated apomorphine injections in a certain cage and repeated Sal in a different one. During the subsequent test the pigeons were injected apomorphine and placed either in the first cage, where the expected latency reduction was observed, or in the second cage, where that latency reduction completely disappeared. (see Carey 1986 for similar results with rats).

A possible explanation for this result is that the emerging CR pecking elicited by the experimental environment (CS), begins to precede to the UR pecking, elicited by the drug (US), which can only act after a certain unavoidable circulatory/diffusional delay. Godoy and Delius (1999) demonstrated that the whole pecking sensitisation could be explained by a classical conditioning phenomenon, in which the CS was a complex stimulus, composed by the apomorphine peck-eliciting effects and apomorphine effects on the perception of the environment. Thus, the conditioning hypothesis proposes that the response sensitisation emerges because a developing CR pecking to the context adds on top of the spontaneous UR pecking directly elicited by apomorphine. This latter hypothesis seems to be well supported by the experimental evidence summarised above.

The apomorphine-context conditioning experimental situation

Different experimental set-up configurations can be used to train pigeons in a classical association between the apomorphine effects and the context. In the experiments reported in the present thesis a basic procedure and a certain apparatus, often used before in our

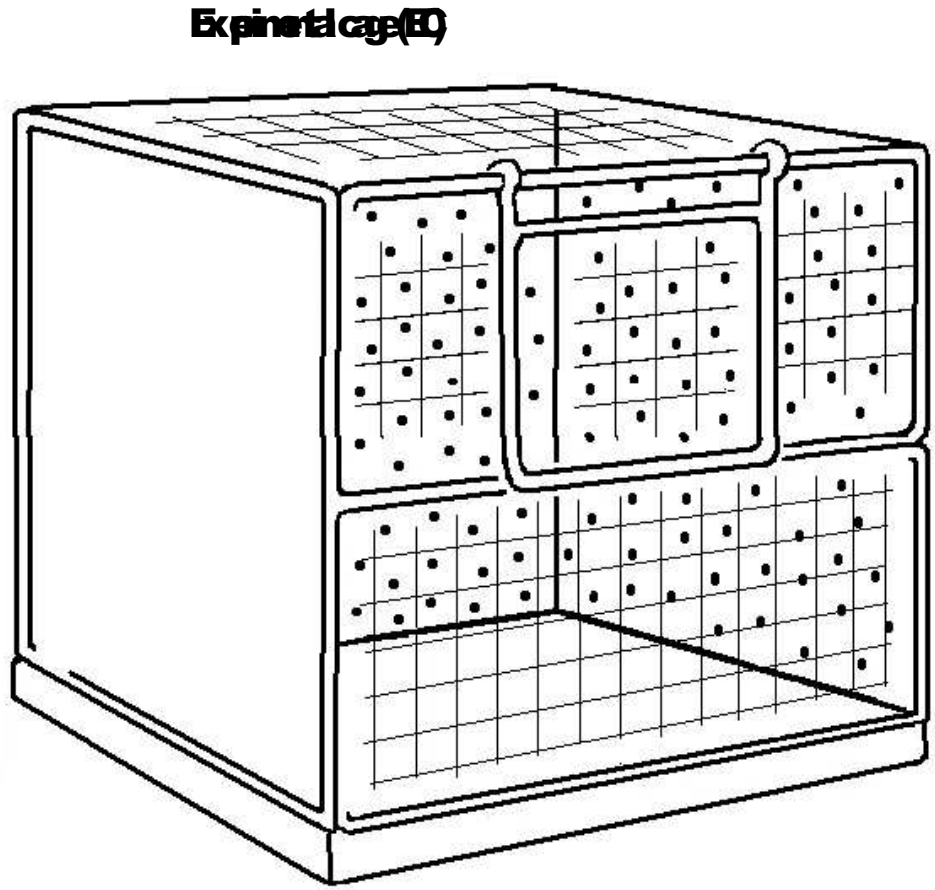


Figure 3.3. A scheme of the visually experimental cage (EC). It consisted of a standard steel grid cages with the back- and side-walls covered with white cardboard panels sprinkled with dark-green dots (taken from Godoy 2000).

laboratory, was used. Briefly, it consisted of injecting pigeons with the corresponding drug solution and immediately placing them in an **e**xperimental **c**age, called the EC, which has certain definite visual characteristics (see below). Here pigeons can be video recorded. The training and test sessions last 20 minutes and the recording data correspond to this time. The apomorphine effects would act as the US thus, the initial pecking response would be the UR pecking directly elicited by the drug. However, with repeated injections in the same cage, pigeons are expected to associate the apomorphine-effects with the context (CS) and consequently, to develop a pecking response conditioned to the cage (CR), on top of the unconditioned pecking, reflecting the sensitisation increment. By the end of training, the total pecking response would be composed of the UR plus the CR. If pigeons are subsequently tested with Sal injection in the experimental cage (EC), a certain CR pecking in response to the cage is expected.

The visually distinctive EC was a standard steel grid cage (the home cages normally used to house the pigeons during the experiment), which had the inner surfaces of its back- and side-walls lined with white cardboard panels randomly sprinkled with dark-green dots

(0.8 mm diameter, about 10 per 100 sq. cm). Figure 3.3 shows a sketch of this cage. This procedure and set-up will be repeatedly referred to in subsequent experiments using the same basic paradigm.

Standard curves for apomorphine-induced sensitisation and saline controls

The sensitisation curves presented in figure 3.2 were obtained by Godoy (2000) using four pigeons per group, and can be taken as an illustrative example. Despite of the fact that the apomorphine sensitisation is a reliable and well-known phenomenon within the above mentioned dose range, it was of value to obtain a standardised sensitisation curve for the apomorphine dose and session time (20 min. per session) used in the experiment throughout this thesis. Such a standard curve could be used as a control for comparisons with the outcome of different treatments in other experiments. Additionally, the result of those treatments must also be compared with the performance of pigeons not receiving apomorphine (a saline injected control group). In figure 3.4, a standard pecking sensitisation curve is presented. It was obtained by averaging the response of 30 pigeons treated with 0.5 mg/kg apomorphine and a standard Sal control curve obtained with the averaged response of 12 pigeons repeatedly injected with saline solution. It can be seen that the pecking response of apomorphine-injected pigeons is significantly stronger than that of Sal injected pigeons in every session (Anova $F_{1,27} = 403.9$, $p \leq 0.05$). The asymptote for 0.5 mg/kg is about 3000 pecks/session, while Sal injected pigeons pecks very rarely (never more than 10 pecks/session) throughout. It is also worth mentioning that the statistical analysis of the sensitisation curve in the Apo group showed a significant inter-session effect ($F_{5,135} = 34.4$, $p \leq 0.05$) by means of two factorial ANOVA.

The data shown in this figure have been collected through several replications of the apomorphine and Sal treatments in the EC, carried out in the course of different experiments. The curve corresponding to 0.5 mg/kg apomorphine treated pigeons also includes data kindly provided by my colleges S. Keller and A. Godoy (unpublished data) belonging to pigeons trained with the same apomorphine dose in the EC. Altogether, the performance of pigeons with these two treatments over a period of 24 months has been averaged to obtain figure 3.4. The large number of experimental subjects and the long period of time seem to be enough to overcome individual differences, seasonal variations and, in general, eventual circumstances that could possibly influence the response. Thus, it can be assumed that these curves are a reliable control for such effects and consequently can be used for comparisons with the results

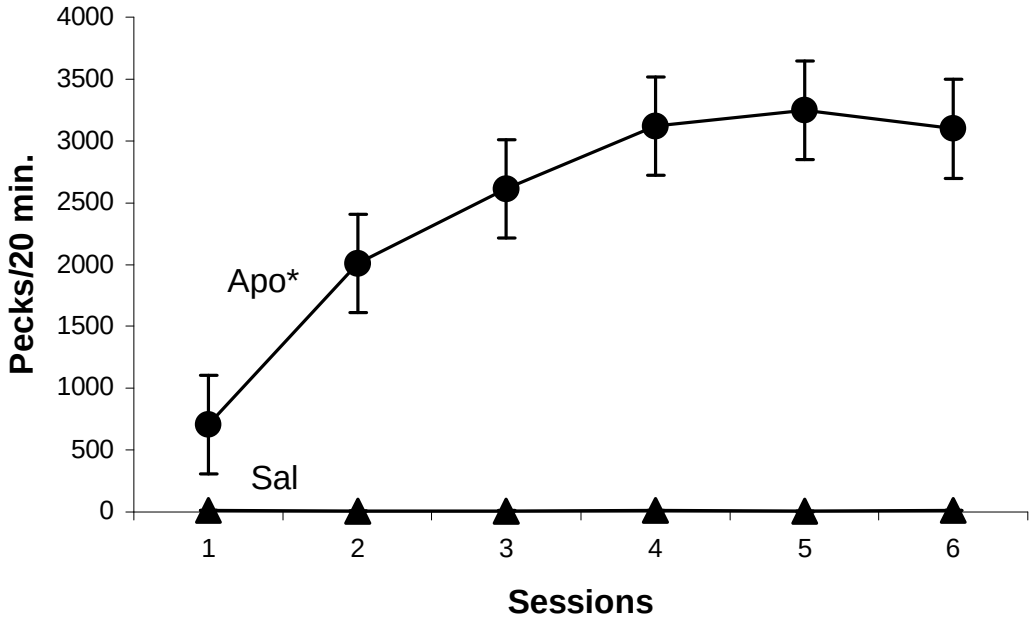


Figure 3.4. Averaged response ±SE of 30 pigeons repeatedly injected with 0.5 mg/kg apomorphine (Apo) and of 12 pigeons treated with saline solution (Sal). The response of the apomorphine treated pigeons increases up to a maximum of about 3000 pecks/session, while that of saline treated pigeons keeps at a level close to zero (* indicates $p \leq 0.05$).

of other experiments reported here. The curve corresponding to apomorphine-injected pigeons will be referred to as the Reference-Apomorphine-Training curve (Ref-ApoTR) and that corresponding to saline injected pigeons will be called Reference-Saline-Training curve (Ref-SalTR) throughout. The use of such "unified control groups", besides standardising the results over many subjects and a long time, has the advantage of reducing the number of experimental animals that would have to be used in unnecessary repetitions of the same treatment. Of course, such a procedure can only be justified for such a consistent, reliable and well-described phenomenon as the apomorphine-induced pecking sensitisation in pigeons.

Besides comparing the performance of pigeons treated with apomorphine and pigeons undergoing other different treatments during the development of sensitisation, it is often relevant to compare the response of pigeons in a subsequent test without drugs, i.e. with saline injection, with that of unsensitised pigeons. It is also a often replicated, and very well known result that pigeons, repeatedly injected with apomorphine until asymptotic sensitisation, respond significantly higher than control pigeons, without apomorphine injection, when they are all subsequently tested with saline injections. Figure 3.5 presents the response of pigeons,

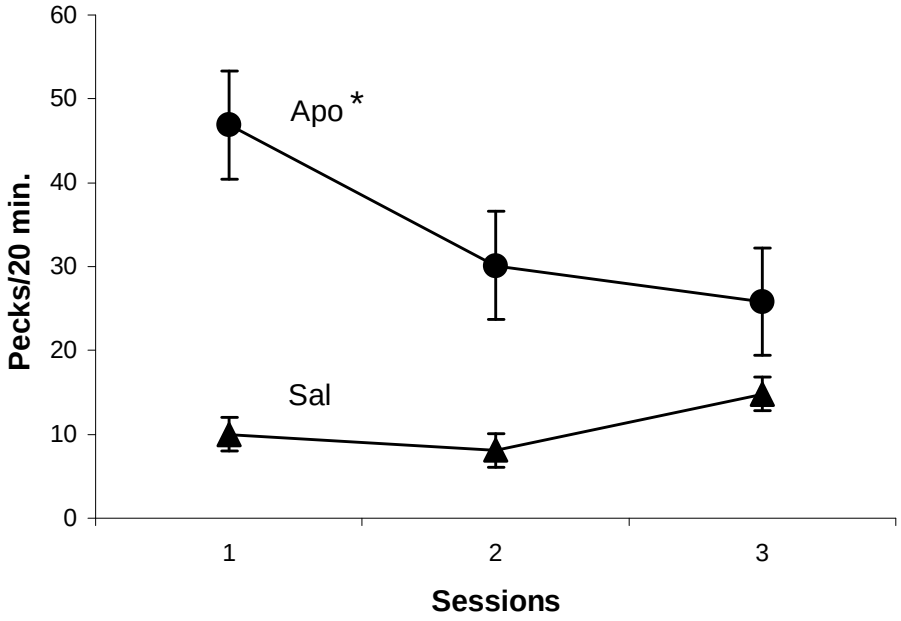


Figure 3.5. Averaged response of 8 pigeons previously trained with repeated injections of 0.5 mg/kg apomorphine (Apo) and of 12 pigeons previously trained with saline solution (Sal), during a subsequent test with saline. Apomorphine trained pigeons responded significantly more than Sal trained pigeons during test sessions (* indicates $p \leq 0.05$).

previously trained with six daily injections of 0.5 mg/kg apomorphine (not shown), during a test with saline, together with the response of pigeons, previously treated with six daily injections of saline, also tested with saline. Both groups were trained and tested in the EC. ANOVA two factorial analysis shields significant differences between Apo and Sal groups ($F_{1,14} = 11.85$; $p \leq 0.05$).

It is worth mentioning that the large difference in the total number of pecks during training and test sessions (about 3000 pecks/20 min. vs. about 50 pecks/20 min.) is mainly due to the compulsive stereotyped behaviour induced by apomorphine in the Apo group. Nevertheless, other secondary effects of the drug may be minimally responsible for the difference in the number of pecks. The dopamine presence in retinal neurones as well as its effect on blood pressure is well known. However, when it is assessed with saline injections, this secondary dopaminergic effect in pigeons seems not to totally block the apomorphine effect acting as US. Despite the difference between levels of response of the Apo group during training and test, the saline control group still responds significantly less than the Apo

group during the first and second test sessions. This difference between Sal and Apo groups is strong enough to suggest that learning processes have taken place in the treated pigeons.

The curve of the apomorphine-treated pigeons in this figure corresponds to the averaged results of 8 birds which were subsequently tested with saline (unfortunately, no saline test data for the other 22 pigeons in that group are available). These data belong to those whose training profile is plotted in figure 3.4. The curve of saline treated pigeons in figure 3.5 corresponds to the averaged test response of the 12 saline-injected pigeons, whose training results are plotted in figure 3.4. The pooled test curves will be considered enough to serve as a standard control for test results. The test curve corresponding to pigeons trained with apomorphine will be called the Reference-Apomorphine-Test (Ref-ApoTS) curve, and that corresponding to pigeons previously trained with saline, will be called the Reference-Sal-Test (Ref-SalTS) curve.

Learning leads to memory

Memory is one of the most extensively investigated cognitive processes. The term memory refers to the ability to reproduce or recount information experienced at an earlier time. Most of the experiments on memory have been carried out with humans and they often involve the use of language. Differently from humans, animal subjects cannot report earlier experiences verbally. However, the existence of memory in animals can be concluded in those cases in which their present behaviour can be predicted from some aspect of their earlier experience. Thus, when an animal's behaviour seems determined by past events, we can infer that some type of memory mechanism is involved in the control of that behaviour (Domjan 1993).

Learning is an enduring change in the response to a particular situation as a result of the previous experience with that type of situation (Domjan 1993). The concepts of learning and memory are very similar and, in fact, such a similarity is well justified. Short and long term learning are not possible without the participation of memory. Moreover, every memory process requires previous learning. One cannot remember things which have never been learned. Memory and learning can be thought of as composed of three basic processes: acquisition, consolidation and retrieval. Acquisition refers to the process in which a subject is exposed to certain kinds of stimuli or information. The consolidation process which refers then to the subsequent period of time in which the information that was acquired is retained. Retrieval is a process in which the information of the original experience is reactivated. While studies about learning focus on the acquisition phase, studies about memory mainly focus on

the consolidation and retrieval processes (Walker 1996). It is well known that with the passage of time, the content of our memory decays. This decrement of the memory might be expressed as “forgetting curves” (Pollmann 1998).

Despite the huge number of experiments developed to learning processes possibly involved in the apomorphine-induced pecking sensitisation in pigeons, (e.g. Godoy & Delius 1999, Wynne & Delius 1995, Burg et al. 1989, Lindenblatt & Delius 1987, Basten-Kreft 1977, Brunelli et al. 1975) there is no study about the long-term retention of the pecking sensitisation induced by apomorphine. The apomorphine-pecking paradigm has been extensively studied and represents a useful tool to evaluate the role of the dopaminergic system in associative learning. It can be used to assess the effects of dopaminergic drugs, to investigate the anatomical elements involved, and is a rather standardised procedure for studying drug-induced learning from an exclusively behavioural point of view. It is thus worthwhile to make every possible contribution to our knowledge about this phenomenon. Probably the possible long-term retention is not the least important. A simple experiment (presented below), which provides a first estimate of the long-term duration of the apomorphine sensitisation was carried out.

Long-term retention of the apomorphine-context association

Experiment 2. This experiment involved selecting pigeons that had been trained in the basic apomorphine-context paradigm described earlier, during various experiments, and testing them with the same task, so that retention at different time periods could be assessed.

Method

Seven pigeons were repeatedly injected with 0.5 mg/kg apomorphine and immediately placed in the EC cage for seven daily sessions. These pigeons were tested for long-term retention at different time intervals. One pigeon was tested after 32 months, 2 pigeons were tested after 29 months, another pigeon was tested after 22 months and 3 pigeons were tested after 17 months. The average time interval was 23.3 months. The different interval times can be grouped into a long and short time interval. The three first pigeons are included in the **l**ong **t**ime **i**nterval group (LTI, one pigeon with 32 months interval, and two other ones with 29 months) with an average interval time of 30 months. The remaining four pigeons are included in the **s**hort **t**ime **i**nterval group (STI, one pigeon with an interval of 22 months and three pigeons with 17

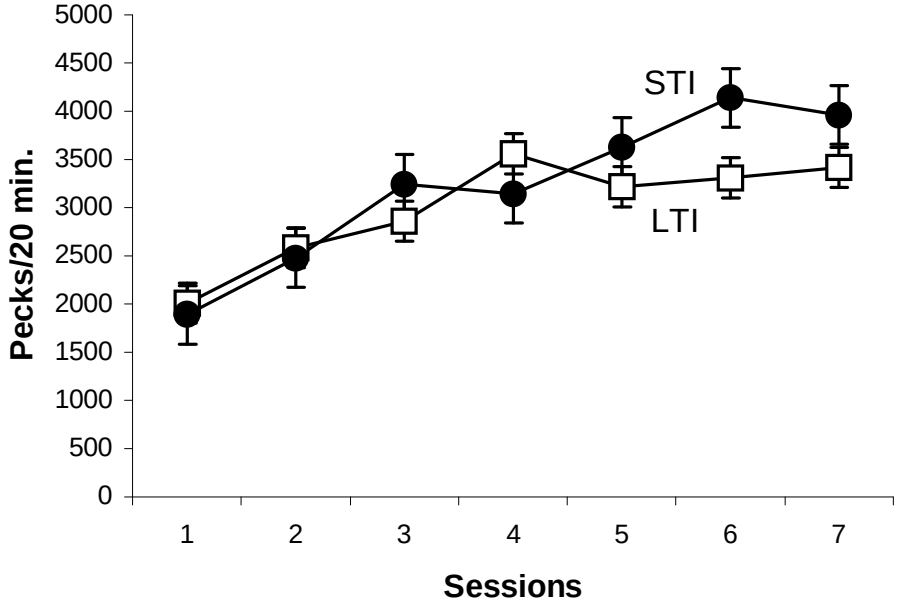


Figure 3.6. Results from Experiment 2 are plotted in averages ±SE. Short (STI) and large (LTI) time interval groups were not statistically different. The STI group consisted of 4 pigeons and the LTI group of 3 pigeons.

months) with an average of 18.3 months interval. For the test, pigeons received previous to test (Pre-test) two saline injections in the EC cage. The main goal was to see whether the subjects would respond to the experimental cage without the influence of apomorphine. After the Pre-test sessions, the pigeons underwent seven daily test sessions with the same apomorphine dose (0.5 mg/kg) in the EC cage. The results of this experiment were analysed using the Mann-Withney test.

Results

Figure 3.6 shows the performance of LTI and STI groups during test. The response of both groups were very similar in the first five sessions and they showed very small differences during the last two sessions. However, no statistically significant differences were found between the STI and the LTI groups. Because the number of pigeons in every group was too small, and no significant differences between them were found, the data of both groups were pooled to compare this with the training data. This allows for a better statistical analysis between training and test.

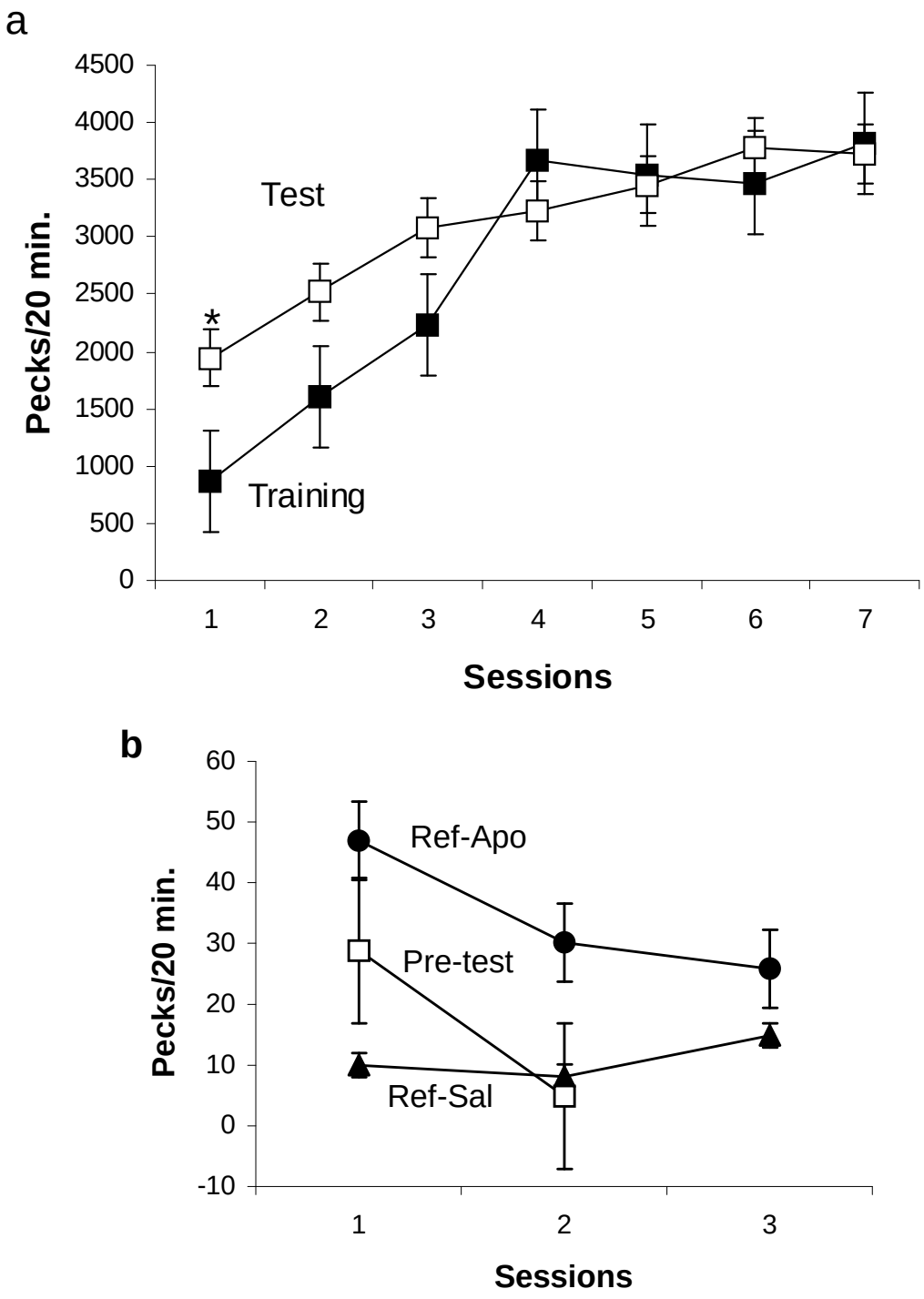


Figure 3.7. Average pecks per session ± SE of long-term memory experiment. **a-** Pecking response from the apomorphine training and Test with an interval time of 23.3 ±2.5 months. Statistical difference was found in the first session (* indicates $p \leq 0.05$). **b-** Pecking response from the Pre-test, apomorphine and saline reference groups (Apo-ref. and Sal-ref., respectively). No statistical differences were found in the Pre-test group compared either with Sal and Apo reference groups.

Figure 3.7 shows the results from training and test respectively with an average time interval of 23.3 ± 2.5 months. The responses during the first three test sessions (figure 3.7a) were higher than during the first three training sessions, although only the first difference was statistically significant (Wilcoxon matched paired test, $Z_{12} = 2.20$; $p \leq 0.05$). The fact that pigeons responded higher in test than in training, when they first experienced the situation, suggests that they still remember and retrieve some of the CR-pecking, although the differences were very small. As the response becomes asymptotic beginning with the fourth session, all differences are lost. Another interesting comparison can be made between the last training session (when the conditioned response is assumed to be fully developed) and the first test session (which represents the first opportunity for the pigeons to express any possible retrieval of the task). The first test response was significantly lower than the last training response. However, as mentioned, it is significantly higher than the first training response. Thus, the retrieval of the apomorphine-context association after an average 23.3 ± 2.5 months period was intermediate between the performance of pigeons without any experience with the task (first training session) and their performance when they have already learned the association (asymptotic response in the last training session).

During the pre-test sessions, the pigeons were injected with saline in the EC cage to see whether they would display any CS pecking without drug after a long retention period (figure 3.7b). Their response during the pre-test can be compared with the response of pigeons tested with saline the day after the termination of a training with repeated apomorphine namely, the Reference-Apo-Test curve presented in figure 3.5. Additionally, it can be compared with the reference response of pigeons trained only with saline, the Reference-Sal-Test curve of the same figure. These comparisons are illustrated in figure 3.7b. As can be observed, the first Pre-test response was higher than the second. However, the response in both sessions was lower than the Ref-ApoTS curve and conversely higher than the Ref-SalTS curve, reflecting an intermediate level of retrieval (differences were not significant). Such a difference is noticeable in light of the fact that the time interval between training with apomorphine and the retrieval of the CS without apomorphine was rather long.

Conclusion

It was shown that pigeons that underwent an apomorphine induced learning process responded consistently higher than pigeons that only received saline. The same was demonstrated by Lindenblatt and Delius (1987) some years ago. Differences in the pigeons'

response were either during training phase (in which pigeons were under the drug effect) or during a posterior test phase (in which every pigeon group received saline). As mentioned before, the difference in pecking responses during training and test phase did not encrypt the still high response of the Apo group due to learning. The fact that the administration of apomorphine could lead to an association between the apomorphine effect and contextual cues was also demonstrated (Lindenblatt & Delius 1987) and supported by several further results (Keller & Delius 2001, Godoy 2000, Godoy & Delius 1999, Wynne & Delius 1995). Nevertheless, there were no experiments focusing memory processes using the apomorphine learning paradigm.

Experiment 2 was the first performed with this particular drug induced learning paradigm that assessed the hypothesis that apomorphine-induced learning is capable of inducing a long-term memory. These results strongly corroborate this hypothesis. Pigeons would learn an association between the apomorphine effects and the context which lasts for at least an average period of two years, a remarkably persistent memory event. In a recent study in mice, it was also demonstrated that a single high-dose apomorphine injection induces a long-lasting sensitisation of the apomorphine-induced stereotyped behaviour, which is context dependent (Battisti et al. 1999). This long-lasting sensitisation persisted for at least 28 days. Pigeons showed the ability to retain a remarkable sensitisation for a much longer period of time. As said, this is a first step in the investigation of the apomorphine-context association in the long-term memory. Further research involving more and different intervals (especially shorter ones) between training and test is required to assess the long-term memory of this association more accurately.

Experiment 2 further contributes to solving the persistent controversy about the true nature of the apomorphine-induced pecking sensitisation, namely, the dichotomy between the above mentioned "pharmacological" and "conditioning" hypotheses to explain the response increment. If pigeons develop pecking sensitisation in a certain environment in response to apomorphine, and that sensitisation can be evoked after a considerably long-term interval (a 23.3 months average interval was used in the above experiment), it can be safely concluded that the role of purely pharmacological factors (non-learning processes) in the sensitisation are minimal, if any. This is especially the case when taking into account that pharmacological changes on the dopamine receptors should have been reverted, after so long a time period. It is widely accepted that behavioural sensitisation does not involve increases in receptor number or affinity (Matsuda et al. 1992, Breese et al. 1987). The pharmacological effects of apomorphine on the dopaminergic receptors last for several hours (Abraini et al. 1994,

Rodriguez & Castro 1991). They mainly involve a decrement effect on the release, turn over and synthesis of dopamine (Rodriguez & Castro 1991). However, apomorphine was found to facilitate the induction and maintenance of LTP processes (Frey et al. 1990) and more recently, slices of nigrostriatals denervated rats were not capable of producing LTP in a magnesium-free media under a high-frequency stimulation (Centonze et al. 1999). Additionally, rats that received a D1-like receptor antagonist, SCH-23390, were not capable of generating LTP in the dentate gyrus of intact brains of rats (Kusuki et al. 1997). Moreover, this mechanism should employ a D1-like dopamine receptor activity for the induction (Kusuki et al. 1997) and a D2-like dopamine receptor activity for the maintenance mechanisms (Frey et al. 1990). These last results support the main hypothesis that in additional to NMDA and non-NMDA mediated effects, dopamine receptor-mediated signals are also necessarily involved in the production of a late, long-lasting maintenance of the LTP process (Frey et al 1990).

Taken together, all these results and the apomorphine-induced learning paradigm involve the activation of the dopaminergic system as a main stimulus. The results presented here are in accordance with earlier data about the dopaminergic activity and long-lasting processes involved in the long-term memory.

In the next chapter, the pharmacological hypothesis will be tested again by means of biochemistry methods and some other pharmacological approaches. The possible dopamine receptor modifications after apomorphine sensitisation, as well as a more accurate place sensitive for this process in the pigeon brain will be tested.

Chapter IV

Dopamine receptor changes consequent upon sensitisation to apomorphine

Introduction

The extensive experience accumulated with the apomorphine-induced pecking sensitisation in pigeons, as well as the experiments described in chapter 3, indicate that such a response sensitisation cannot be explained in terms of purely pharmacological processes. Learning must be strongly involved. For example, it does not seem probable that pharmacologically determined latency reduction, as a consequence of repeated apomorphine injections, will be expressed only in a particular cage, by the same subjects on the same day, as Wynne & Delius (1995) nevertheless reported. Learning, on the other hand, can explain such an outcome if we assume that pigeons learns an association between the apomorphine effects and a particular cage. However, as mentioned, there is no reason to postulate that learning and pharmacological processes mutually exclude each other. Furthermore, even when the whole pecking sensitisation can be accounted for by learning (Godoy & Delius 1999), it has to be brought about by neurobiochemical changes in the central nervous system.

As discussed in the previous chapter, the possibility that apomorphine modifies dopaminergic receptors (either in their total number or in their affinities for dopamine) deserves further investigation. Apomorphine facilitates the induction and maintenance of LTP processes (Centozone et al. 1999, Kusuki et al. 1997, Frey et al. 1990). It was crucial to determine whether the apomorphine effects on the dopaminergic synapses were related to induction of such possible learning mechanism as LTP processes. Or more directly, it modifies the dopaminergic synapses, increasing the receptors number or changing their affinity for the endogenous dopamine. The present chapter is devoted to the exploration of apomorphine-induced pecking sensitisation from a biochemical and anatomical, rather than a behavioural, point of view. Naturally, being an apomorphine a dopaminergic analogue, the dopaminergic system is the best candidate for a first exploration of possible biochemical changes, emerging after a sensitisation training. Two experiments are presented in this chapter. In experiment 3, binding assay

was used to assess possible changes in the biochemical parameters, number and affinity of dopamine receptors after an apomorphine-training. Experiment 4 explores the participation of two brain structures, the nucleus accumbens and the striatum (paleostriatum augmentatum and paleostriatum primitivum) in the apomorphine sensitisation of pigeons.

Biochemical modifications of the dopamine receptors.

Experiment 3. Stereotyped behaviour refers to actions that occur repetitively, with very little variation, and which have apparently no wear upon the full function for the organism. As explained in chapter III, apomorphine induces stereotypes in a wide variety of species such as snails, toads, fishes, tortoises, mice, rats and humans (Glagow & Ewert 1997a, 1997b, Szechtman et al. 1987, Ljungberg & Ungerstedt 1977, Andersen et al. 1975, Fekete et al. 1970). Frequently, it induces oral stereotypes, like chewing or pecking. In rats, apomorphine induces repetitive sniffing, licking, chewing, and hyperactivity in running and rearing (Havemann et al. 1986). It induces increased climbing in mice (Cabib & Puglisi-Allegra 1988, Kendler & Davis 1984) and guinea pigs (Frommel et al. 1965), sniffing in cats (Motles et al. 1989) and rabbits (Hill & Horita 1972, Harnack 1874) and chewing in dogs (Nymark 1972), monkeys (Shintomi & Yamamura 1975) and humans (Lal 1998). In pigeons and chickens, low doses of apomorphine induce bouts of repetitive pecking (Dhawan et al. 1961, Dhawan & Saxena 1960).

As already mentioned, the effects of apomorphine on pigeons change after a chronic treatment with this drug. When a certain dose is repeatedly injected into pigeons, the total pecking response elicited by each injection increases up to a dose-dependent maximum. The pecking response thus sensitises up to a certain asymptotic level (Wynne & Delius 1995, Basten-Kreft 1977). Such chronic treatments with some drugs can induce a chronic stimulant intoxication syndrome in humans and animals. Ellinwood et al. (1973) proposed that it consists of two main stages. An earlier stage (about 1-2 weeks) is characterised by increased suspiciousness and repetitive behaviour in humans, and intense stereotypy in animals. A later stage (2-3 months) is, in humans, characterised by a psychotic-paranoid panic behaviour or by a hyper-reactive, fear-like state, often with hallucinations and, in animals, by tolerance to dopamine, stereotyped behaviours, and an increase in bizarre behaviour (e.g. the hyper-reactivity to stimuli).

Behavioural experiments that use apomorphine chronic treatments, normally do not last more than six to ten days (Godoy 2000, Godoy & Delius 1999, Wynne & Delius 1995, Burg et al. 1989, Lindemblatt & Delius 1987). Therefore in these experiments, the animals may never progress past the earlier stage, as proposed by Ellinwood (1973).

Other than apomorphine, additional dopaminergic drugs also have been found to induce stereotypes, although with lower potency and higher toxicity than apomorphine. Cocaine and amphetamine are also dopaminergic agonists, but they act indirectly. Cocaine acts by blocking the dopamine re-uptake from the synaptic cleft, and amphetamine, besides blocking the dopamine re-uptake, also stimulates the neurotransmitter release from the pre-synaptic cell. Apomorphine acts directly on the D1- and D2-like dopamine receptors. In rats, the chronic treatment with cocaine, amphetamine or apomorphine, produces sensitisation of the locomotor activity. As mentioned, chronic apomorphine produces sensitisation of the stereotyped pecking of pigeons and chicks (Brunelli et al. 1975). However, cocaine does not and amphetamine only weakly induces stereotyped pecking (Schaal et al. 1995, Goodman 1981, Kraemer et al. 1997, Cheng et al. 1975). In birds, relatively low doses of cocaine (0.5 – 1.0 mg/Kg body weight) increase the locomotor activity, but higher doses (2.0 mg/kg), induce retching, just as do doses of apomorphine (Delius, personal communication). All the doses of amphetamine tested in pigeons (0.5 – 4.0 mg/kg) induce immobility and calmness (Delius, personal communication).

The mechanisms underlying the augmentation of the stereotypy by repeated administrations of direct dopaminergic agonists in animals is still unknown. In order to explain this phenomenon, several mechanisms have been proposed. The most simple explanation invokes post-synaptic alterations in the number or affinity of dopamine receptors. However, there are a number of different ways by which the efficacy of a synaptic connection can be modified. Changes can occur in the pre-synaptic or in the post-synaptic membrane, as well as in the synaptic cleft. Possible pre-synaptic modifications would involve the rate of synthesis or the rate of release of the neurotransmitter, the growth of new axonic branches or the formation of new active ones, and the inhibition of the transporter mechanisms, which allow the re-uptake of the neurotransmitter molecules. In the case that pre-synaptic receptors exist, their number and/or affinity can change. Possible post-synaptic alterations would include modification in the numbers or affinity of the corresponding receptors, and increments of the dendritic branches' diameter, allowing faster action potentials. Modifications to

the synaptic cleft would include the inhibition of the metabolising enzymes, which in turn allows for a longer receptor exposition time to the neurotransmitter. Bearing in mind that the direct agonist apomorphine effectively induces stereotypic pecking behaviour, while the indirectly acting amphetamine and cocaine do not, it is of interest to examine how the dopaminergic pre- and post-synaptic receptors are affected by chronic treatment with this drug. For such a study, the radioligand binding assay technique is an adequate tool.

The radioligand binding assay technique

Basically, this technique clarifies the physiochemical interaction between a radioactively labelled receptor ligand and the receptors (pre- and post-synaptic) present in the relevant tissue preparation. Some important criteria must be satisfied to carry out a binding assay: The radioligand should have demonstrable biological activity as an agonist or an antagonist, and should also have a high specific radioactivity. Low receptor concentrations cannot be determined if the specific radioactivity is lower than 5 or 10 Ci/mmole. It should also have high affinity and selectivity for the receptor sites under study (e.g. Spiperone has a high affinity and selectivity for the D2-like receptors and a very low affinity for the D1-like and for serotorinergic receptors). Finally, since there is only a finite number of receptor sites, the binding of the radioligand to the receptor preparations must demonstrate saturability. Another important issue is to select tissue which is rich in the relevant receptors. It is very well known that the basal telencephalon of pigeons, like that of mammals (rats, monkeys or humans), is rich in dopaminergic receptors (Richfield 1987). For that reason, in the following experiment, the basal telencephalon of pigeons was used as the preparation to perform binding assay on dopaminergic receptors (see below).

Equations and parameters of the binding assay technique

Basic thermodynamic principles allow the derivation of simple equations, which can be applied to describe the behaviour of drug-receptor systems during a binding assay with radioligands. Most of the concepts behind these equations derive from the Mass Action law, from the pharmacological theory of A.J. Clark, and from the classic Michaelis-Menten analysis of enzyme substrate kinetics.

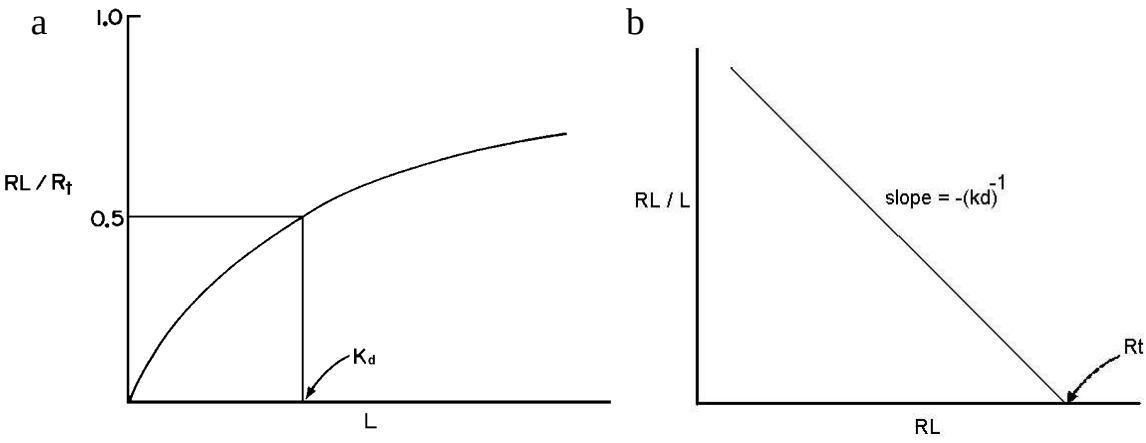


Figure 4.1 a) RL/Rt is the proportional relation of the complex ligand-receptor (RL) out of the total receptor concentration (Rt); L is the free ligand. At equilibrium, the dissociation constant (Kd) is defined by the a value of concentration of half of the receptors occupied by the ligand (see main text, equation 2). **b)** Scatchard plot. RL / L is the proportion of ligand bound to the receptor from the total ligand concentration (L). The slope of the line is the negative reciprocal of the dissociation constant. When all the receptors are occupied by the ligand (saturated condition), the concentration of the complex RL is equal to the total amount of receptors Rt.

The simplest mechanistic assumption that can be made about the interaction of a radioactive ligand L with a receptor R is that a single molecule of L interacts reversibly with a single molecule of R, to form a complex RL. The equation of the law of the mass action that describes this situation is: $L + R = RL$ (eq. 1). Each molecular interaction is assumed to be independent of other interactions. Now, if L, R and RL are thought in terms of mol/litre units and not as a single molecules at equilibrium, a direct consequence of the law of the mass action results: $(R * L) / RL = Kd$ (eq. 2), where Kd is the equilibrium dissociation constant of the complex and R, L and RL represent the concentrations of free-receptor, free-ligand and bound-ligand, respectively. The total concentration of receptors Rt is equal to R plus RL. Thus the corresponding equation is $(Rt – RL) * L / RL = Kd$ (eq. 3).This can be re-arranged as $RL / Rt = L / (Kd + L)$ (eq. 4). The ratio RL / Rt represents the fraction of total receptor occupied by the ligand. At half maximal occupancy of the receptor RL / Rt is = ½ and Kd = L. Hence, the concentration of L required for half-maximal occupancy of the receptor is equal to Kd. Additional re-arrangement of the former equation gives $RL = (Rt * L) / (Kd + L)$ (eq. 5). The later equation is the familiar hyperbolic function in which RL = 0 when L = 0, and RL approaches Rt, when L is very large. This equation is especially useful in binding

studies to derive the Kd and the number of binding sites, Rt. Empirically, a range of different radioligand concentrations is added to a fixed receptor concentration. The level of binding that approaches asymptotically at high ligand concentrations is Rt, and the concentration of free ligand that elicits a level of binding equal to Rt / 2 represents the Kd (Figure 4.1a and 4.1b).

The assumptions made for this analysis are that the reaction is a simple bimolecular reaction (eq. 1) and that the binding is measured at equilibrium. A useful rearrangement of equation 3 is $RL / L = (Rt – RL) * Kd^{-1}$. This is the equation derived by Scatchard (1949). Thus a plot of the ratio of bound to free ligand RL / L, versus the concentration of bound ligand RL, gives a straight line with a slope equal to K_d^{-1} that intercepts the abscissa at R_t. The main advantage of the Scatchard plot over the analysis using equation 5, is that the data are transformed to a linear function, something particularly valuable in binding systems that have a high level of non-specific binding. In such systems, measuring binding is difficult at high radioligand concentrations because the relative contribution of the non-saturable, non-specific "background" binding is very large. This makes it difficult to estimate the R_t, and consequently the K_d, with equation 5. By contrast, using the Scatchard analysis, data can be obtained at low or intermediate concentrations of L and the linear plot can be extrapolated to estimate R_t and K_d.

Radioactivity is measured by means of a scintillation counter, which gives radioisotope degradation per minute (dpm) values. The measured dpm values are converted into fmol/mg of protein by

$$B \text{ fmol/mg prot.} = (X \text{ nmol} * 10^{-6} \text{ fmol/nmol}) / P \text{ mg}$$

where B is the receptor concentration in fmol per milligrams of protein, P is the protein concentration in the sample and X is the measured receptor concentration in the sample. The latter is obtained using the following equation

$$X \text{ nmol} = (1 \text{ mmol} * 1 \text{ µCi} * X \text{ dpm}) / (Act \text{ µCi} * 2.22 \text{ dpm} * 10^{12})$$

where X is the amount of desintegration per minute (dpm) measured in the sample, Act is the radioisotope activity in microcurie (µCi) and 2.22 is a constant that converts µCi to dpm. The rest of the factors in the formula come from unit transformations.

Tissue preparation

As mentioned before, in this experiment, the binding assay technique was performed in the pigeons' basal telencephalon. The purpose of such an experiment is to assess possible changes in the numbers and/or affinities of the D1- and D2-like receptors, as a consequence of a chronic apomorphine treatment. The basal telencephalon was chosen as the studied tissue preparation because of its known richness in D1- and D2-like receptors. This structure has been found to contain D1- and D2-like dopamine receptors in many different species such as turtles, pigeons, quails, rats, cats and monkeys (Richfield 1987). This was determined using several techniques such as binding assay, autoradiograms with specific radioligands, detection of dopamine receptors mRNAs by *in situ* hybridisation, and immunohistochemistry with monoclonal antibodies against the different dopaminergic receptor types.

Autoradiograms have been useful in the anatomical studies of regional and cellular distribution of viable ligand recognition sites for the two pharmacological defined families of dopamine receptors. Most of the studies used the prototypical antagonist SCH-23390 to assess D1-like binding sites. Additionally, they used sulpiride, spiperone and raclopride among others, to determine the D2-like dopamine receptor binding sites. In pigeons, dopamine receptors have been found in the basal ganglia (Durstewitz et al 1998, Karle et al. 1996, Ball 1995, Richfield 1987). The pigeon's brain contains D1-like dopamine receptors in the paleostriatum augmentatum, lobus parolfactorius, paleostriatum primitivum, nucleus intrapendicularis, ventral pallidum, bed nucleus of the stria terminalis and nucleus tegmenti pedunculopontis, pars compacta. D2-like dopamine receptors are also present in the same regions as D1-like dopamine receptors, with some small differences in their densities. (Ball 1995, Richfield 1987).

Method

Eight pigeons were repeatedly injected with 1.0 mg/kg apomorphine, on daily sessions for 9 days in their home cage. Another eight pigeons were injected with saline under the same conditions. Four pigeons treated with apomorphine and four with saline were used to measure D1-like receptors. The remaining ones were used to measure D2-like receptors. Two days after the end of treatment, animals were deeply anaesthetised with

Nembutal (sodium pentobarbital). Subsequently, intracardiac perfusions were performed. The brains were quickly removed and the basal telencephalon immediately dissected. The lamina medullaris dorsalis (which forms the dorsal and lateral boundaries of the paliostriatal complex) served as a natural boundary for removal of the basal telencephalon. The tissues were weighed and then frozen to –80°C, until the day when they were processed for binding assay.

To perform the binding assay, tissues were homogenised in 50 mM tris-HCl (1:50 w/v) buffer (pH 7.8), containing 1 mM MgCl2 2mM CaCl2 120 mM NaCl and 5 mM KC,l in a 30 ml glass homogenisator with a teflon piston for 10 min. To avoid degradation of receptors by proteolitic activity of the proteases present in the solution, the homogenisation solution was maintained at 4°C or lower. The homogenate was centrifuged at 48,000 g for 10 minutes and the pellet was washed in the same buffer and re-centrifuged under the same conditions. The final pellet was re-suspend in 50 mM tris-HCl buffer (pH 7.4), containing 1 mM MgCl2 2 mM CaCl2 120 mM NaCl and 5 mM KCl, to yield a final concentration of 1 mg/ml. To determine the [^{3}H]SCH-23390 to D1-like receptor binding saturation curve, 300 µl of membrane suspension were incubated by triplicate for 30 min at 37°C, in the presence of [^{3}H]SCH-23390 (Du Pont, 70,3 Ci/mmol) with 8 different concentrations ranging from 0.0156 nM to 2 nM. For the [3H]spiperone to D2-like receptor saturation curve, the same procedure was used with [^{3}H]spiperone (Du Pont, 14,6 Ci/mmol). This time 7 different concentrations ranging from 0.0156 nM to 1 nM were used. The final volume was 50µl. To terminate the binding reaction, the solutions were immersed in ice. For the assessment of non-specific binding, 1µM cold SCH-23390 (D1) or 1µM (+)-butaclamol (D2) (both from RBI), were added to the incubation medium. Immediately after terminating the binding reaction and separating bound from free ligand, the tubes were centrifuged at 16,000 g, 4°C for 6 min, the supernatants removed by aspiration, and the non-rinsed pellet-containing tips of the plastics tubes were cut off (with a heated wire) and placed in scintillation vials. After the addition of scintillation liquid (SLD-41, Spolana), vials were shaken for 2 hrs, allowed to equilibrate overnight and counted with a Beckman LS6000SE scintillation counter during 10 minutes for each scintillation vial.

Protein values were determined according to the Lowry method using a protein kit from Sigma. Data from saturation studies were analysed using a specific software (Enzfitter, Elsevier-Biosoft). For statistical assessments, the non parametric Mann Whitney test was used.

Results and discussion

As expected, pigeons injected with saline displayed very few pecks throughout the treatment. Conversely, pigeons injected with apomorphine exhibited a strong stereotyped pecking reaction, which increased over sessions until it reached an asymptotic level (data not shown, compare Fig. 3.4). The asymptote was reached by the fourth session and remained asymptotic until the end of treatment. Thus, the apomorphine-induced pecking response of these pigeons can be considered fully sensitised for the binding assay.

The results of the binding assay with both [3H]-SCH23390 and [3H]-spiperone yielded readily saturable relationships for both groups and showed high affinity in the pigeons' basal telencephalon. Figures 3.4a and 3.4b show representative results for one pigeon of each group. Saturation studies showed that the affinity of [3H]-SCH23390 and [3H]-spiperone did not differ significantly between apomorphine and saline treated groups (Mann Whitney U test, $Z_6 = 2.31$, $p \leq 0.05$). Table 4.1 shows the results of this analysis. Saturation curve and Scatchard analysis for representative pigeons of every group are shown in figure 4.2.

The amount of binding sites for D1-like receptors was higher in apomorphine treated pigeons than in saline treated ones, although this difference did not achieve statistical significance (figure 4.2a and b, and table 4.1). The amount of D2-like receptors was smaller in the apomorphine treated pigeons than in the saline treated ones, but again, this difference was not significant (figure 4.2c and d, and table 4.1). However, when the relationship of D1-like to D2-like receptor amounts (D1/D2) between groups is analysed, the difference appears. It is noticeable that, even when the number of D1- and D2-like receptors is not significantly different between groups, the ratio D1/D2 is. The apomorphine treated pigeons had a significantly higher D1/D2 ratio than the saline treated ones. This result suggests that a chronic apomorphine treatment capable of inducing pecking sensitisation is also capable of producing biochemical changes in the dopamine receptors. Such changes remain unnoticed when only the receptor numbers are compared, but become evident when the proportion D1/D2 is compared with that of a control. Several explanations may account for the fact that differences in receptor number between apomorphine and saline treated pigeons are so minimal. For example, it is possible that the apomorphine-induced sensitisation would

	D1-like receptors		D2-like receptors		RD1/RD2
	Kd (pM)	R(fmol/mg prot)	Kd (pM)	R(fmol/mg prot)	
Apomorphine	462.85	169.05	62.64	112.9	1.5*
Saline	362.0	146.34	53.93	132.84	1.1

Table 4.1. The receptors dissociation constant values (Kd) and the receptor concentrations (R) of apomorphine and saline treated pigeons. The two families of dopamine receptors were compared between both groups. No significant difference was found either in the receptor concentration, nor in the Kd values. The ratio RD1/RD2 of receptors concentration, were statistically different between apomorphine and saline treated groups (* indicates $p \leq 0.05$).

produce only a discrete receptor change so that the amount of tissue assayed turns out to be critical.

It is possible that only a few dopaminergic synapses are modified. However, in this study, smaller amounts of tissue could not be taken because it is not clear which areas of the basal telencephalon are directly involved in the changes due to apomorphine treatment. One could speculate that these synapses could be modified not only because of the apomorphine action on them, but also because the enhancement of such dopaminergic signals does not depend only in the apomorphine stimulation. Apomorphine should affect the receptor amount in all dopaminergic synapses and not only these related to the stereotyped pecking response. It cannot be ruled out that the action of apomorphine on the dopaminergic receptors also involves elements related to synaptic plasticity. Since pigeons injected with apomorphine were placed into their home cages, the cages themselves could serve as a contextual stimulus for the apomorphine-induced learning.

Although the phenomenon of apomorphine-induced pecking sensitisation has been repeatedly proven to be based on associative learning (e.g. Godoy & Delius 1999, Wynne & Delius 1995) biochemical changes as a consequence of training has never been studied. The assessment of such changes was essential in the apomorphine-context conditioning experiment described in chapter III. Experiment 3 presents for the first time, evidence of modifications in the receptor parameters of dopaminergic receptors as a consequence of a chronic apomorphine treatment in pigeons. At this point, it is

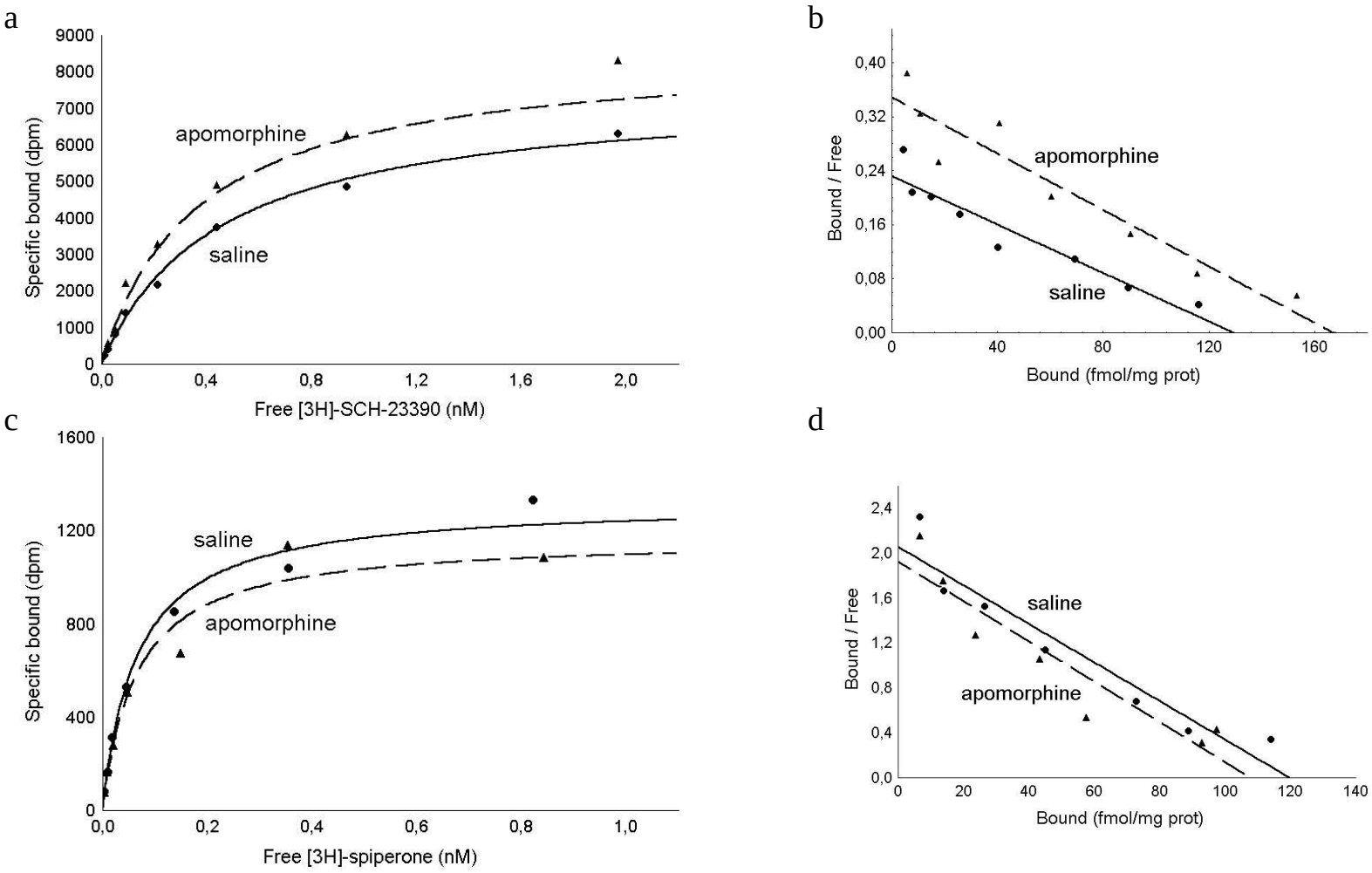


Figure 4.2 Representative pigeons from saline and apomorphine groups are plotted. **a)** and **c)** Saturation curve of the binding assay for D1- and D2-like receptors respectively. **b)** and **d)** Scatchard analysis of the D1- and D2-like receptors binding assay respectively.

impossible to determine whether the reported changes are part of the molecular mechanism responsible for the apomorphine-context association, or if they are a concomitant phenomenon. Additionally, the differences between apomorphine and saline treatments, namely the differences in the D1/D2 ratios reported here, were small. In order to investigate these differences in more detail, technical adjustments must be pursued in future research.

Local administration of apomorphine into the pigeon's brain.

Experiment 4. It is well known that the nucleus accumbens and striatum (which are part of the basal telencephalon) studied in the previous experiment, play an important role in motor activation and sensitisation processes. The nucleus accumbens is involved in the control of several processes such as locomotion (Mathé et al. 1998), stereotypes (Gargiulo 1996), motivation (Ikemoto & Panksepp 1999), reward (Koch et al. 2000b, 1996, Miyazaki et al. 1998), learning (Di Chiara et al. 1999) and memory (Setlow & McGaugh 1998). The nucleus accumbens shares most of these properties with the striatum (Cory-Slechta et al. 1999, Cousins et al. 1999). The dopamine present in both areas has been found to play an important role in locomotor activity (Schmidt 1998). Therefore, it was of interest to investigate the possible participation of these two brain areas in the pecking sensitisation of pigeons. In the following experiment, cannulations into nucleus accumbens or into the striatum are used to allow for the intra-cerebral administration of drugs. In this way, it was possible to assess how the localised action of apomorphine into these areas influences the subsequent performance of pigeons in the apomorphine-induced pecking sensitisation.

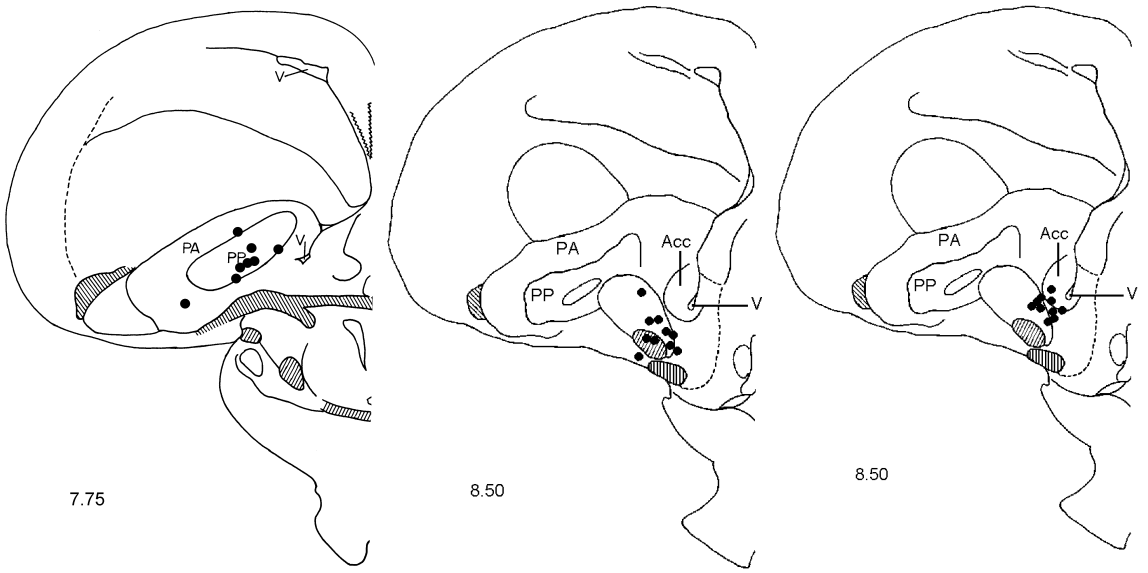
Method

One group of pigeons was implanted with cannuli into the nucleus accumbens in both brain hemispheres. A second group was implanted with cannuli into the caudal striatum (paleostriatum augmentatum and paleostriatum primitivum) bilaterally. In this experiment, pigeons were trained with repeated intramuscular (i.m.) injections of apomorphine or saline in a distinctive experimental cage (EC cage, shown in figure 3.4, chapter III). Subsequently, they were tested with intracranial (i.c.) administrations of apomorphine or saline in the same cage.

Cannuli. Pigeons were anaesthetised with an initial i.m. injection of 0.1 ml Kemint (Alvetra, ketamine chlorhydrate 100 mg/ml) and 0.02 ml Rompun (Bayer, xylazine 23 mg/ml) per 100 g of pigeon's body weight. The anaesthesia was maintained with additional 0.1 ml i.m. injections of Kemint every 20 min. intervals. The pigeon's head was held by means of a stereotaxic apparatus, while implanted with two guide cannuli (stainless steel tubing, 23 gauge, 13mm long for nucleus accumbens and 11 mm long for striatum) with their bevelled tips aimed at locations 2 mm above the right and the left nucleus accumbens, or in the middle of the caudal striatum. The centres of the latter structures were estimated to be located at the following co-ordinates of the Karten & Hodos (1967) pigeon brain atlas: A 8.5, D 7.5, L 2.0 for the nucleus accumbens and A 7.75, D 8.5, L 2.75 for the caudal striatum. The cannuli were fixed to the skull with acrylic cement and kept occluded with removable stainless steel pins (30 gauge, 13 mm long for nucleus accumbens and 11 mm long for striatum). After surgery, pigeons were allowed to recover for a week.

Procedure. The nucleus accumbens group (NA), consisting of twelve pigeons that had no previous experience with drugs. This group was trained with 0.5 mg/kg i.m. apomorphine during four daily sessions and then tested with i.c. administrations into the nucleus accumbens of a 20 µg/µl apomorphine solution, 1µl per hemisphere, during three daily sessions. Immediately after each drug administration, pigeons in this group were placed into the EC, for 20 minutes, where their behaviour was filmed. The apomorphine concentration, as well as the basic procedure were both based on the work from Lindemblatt (1986).

The striatum group (ST), consisting of four pigeons, had previous experience with apomorphine. These pigeons had participated in another experiment in which they had been trained with six daily i.m. injections of 0.5 mg/kg apomorphine plus i.c. saline administration in the EC cage. They were tested with three daily i.c. and i.m injections of saline also in the EC cage (these pigeons took part in experiment 10 presented in chapter VII, where they are included into the "group apomorphine"). On the day following the last test session of experiment 13, these pigeons received one session with i.m injection of 0.5 mg/kg apomorphine and i.c. administration of saline to refresh the apomorphine-induced pecking sensitisation achieved during the training of experiment 10. This group underwent three daily sessions with i.c. administrations of a 20 µg/µl



Striatum **Control** **Nucleus accumbens**

Figure 4.3 Location of the cannuli tips in the pigeon brain according to Karten and Hodos (1967). The cannuli were placed into the striatal zone (paleostriatum augmentatum PA, and primitivum, PP), nucleus accumbens (Acc) and for the control group in the area of the fasciculus prosencephali lateralis, fasciculus diagonalis Brocae and tractus quintofrontalis. Only one side of the brain hemisphere containing the cannuli location of both side are shown.

apomorphine solution into the striatum, 1µl per hemisphere. Immediately after each injection, pigeons were placed into the distinctive cage for 20 minutes and filmed. For the i.c. drug administrations, pigeons were held manually and the occlusion pin of the relevant cannula was removed. A 30-gauge, 15 mm-long (for NA) or a 12 mm-long (for ST), stainless steel injection cannula attached to a microsyringe (Hamilton 10 µl) was introduced through the guide cannula. To allow for diffusion, 1µl solution was gradually injected over 2 minute periods. Because apomorphine is easily oxidised (and the oxidised form is not biologically active) solutions of this drug had to be carefully prepared. This was done as follows: R-(-)-apomorphine HCL (from ICN) was dissolved in 0.1% sodium disulfit (as an antioxidant) and exposed to ultrasound until it was dissolved (about 1 hour). The resulting solution was always maintained in an oxygen free atmosphere with a rubber cap covering the tube. This cap was perforated before each apomorphine administration through the injection cannula. The apomorphine used

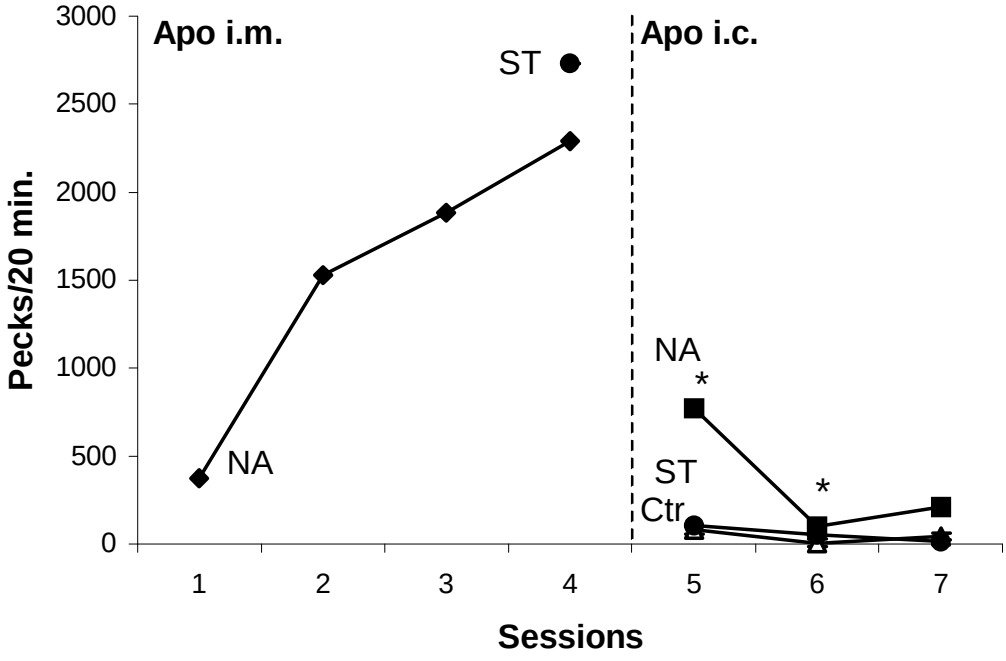


Figure 4.4. Average pecking responses of i.m. and i.c. injected pigeons. The 11 responding pigeons of the group nucleus accumbens (NA) received i.m apomorphine. This group was subsequently divided into two subgroups: n.acc and control (Ctr) according to the i.c. injection sites as revealed by histology. The 4 pigeons of group striatum (ST) had received six apomorphine injections, followed by three saline injections, during a former experiment (experiment 10, chapter VII) and one "refreshment" i.m. apomorphine injection in this experiment, the only session plotted in this figure. The pecking response of group NA was statistically different from that of group Ctr in the fifth and sixth sessions (* indicates $p \leq 0.05$)

for i.m injections was a racemic mixture (from Teclapharm) in ampoules containing a special antioxidant medium (sodium disulfite 0.1mg/ml and methyl-4-hydroxybenzoate 0.12 mg/ml). As is normally the case for i.m. injections, the corresponding concentration was made diluting the apomorphine solution with saline (0.9% NaCl).

Histology. After the experiment was completed, the pigeons were anaesthetised and injected through the cannula with 0.1% cresyl violet 1 µl per hemisphere. They were then transcardially perfused with saline solution and then with a 4% formaldehyde solution. The brains were removed and fixed in the same formaldehyde solution. Before histology, they were placed into a formaldehyde solution with 30% saccharose for one

day. The brains were then mounted and frozen in a cryotome and slices were cut at 40 µm intervals. Every 5th section around the injection site was mounted. Microscopic inspection of these sections with a 10x magnifying lens ascertained the site locations. These locations were transferred to standard sections taken from the pigeon brain atlas (Karten & Hodos 1967).

Results and discussion

Seven pigeons of the NA group had both cannuli placed in the nucleus accumbens or in its immediate vicinity as confirmed by the histology of their brains. Four pigeons of this group had at least one of the injection sites out of the nucleus accumbens and were used as controls. One pigeon of the same group had to be discarded because it never pecked when injected i.m. apomorphine (some few pigeons do not peck in response to apomorphine for probably genetic reasons). The four pigeons of group ST had the injection sites well located in the caudal striatum or in its vicinity. The results of the histology are shown in figure 4.3. For simplicity, all the injection places are shown in only one brain hemisphere for each group. Black dots represent the centre of the injection place for every cannuli implantation.

Figure 4.4 shows the performance of pigeons during the training and test phases of experiment 4. During training with i.m. apomorphine injections, pigeons of group NA exhibited a normal sensitisation of the pecking response. Pigeons of the group ST were already sensitised. The single dot (full circle) plotted in figure 4.4 shows the refreshment of this group with apomorphine i.m. injection one day after to be part of the experiment 13, as mentioned before.

For the test phase in which pigeons received i.c. administration of apomorphine, the results of group NA were subdivided into two subgroups: NA corresponding to those pigeons effectively injected into the nucleus accumbens and the control (Ctr), which had the injection sites outside of the nucleus accumbens and outside of the striatum, as revealed by histology (figure 4.3). Since all the pigeons in the ST group had the cannuli located in the correct site, no pigeon in this group could be used as a control.

The response of pigeons with the cannuli placed bilaterally in the nucleus accumbens was significantly higher than that of the control group in the fifth and sixth sessions in test (Mann Whitney U test, $Z_{10} = 2.27$ and $Z_{10} = 2.00$ respectively, $p \leq 0.05$).

No other significant differences were observed. Pigeons injected with apomorphine in the striatum responded at control levels during test. Despite the larger amount of training sessions with i.m. apomorphine given to the ST group (six sessions in the previous experiment and one as refreshment in this experiment versus four session for NA group), the response during the test was not statistically different from control group.

General discussion

The apomorphine-induced pecking sensitisation is undoubtedly the outcome of a drug-induced learning process, namely the association between the apomorphine effects and the context. Keller and Delius (2001) found in our laboratory, that pigeons daily injected with i.m. apomorphine injections and placed in a completely different cage each time display the apomorphine-induced stereotypic pecking bouts, but do not develop sensitisation of this response. Rather than that, the total pecking stayed almost constant throughout. Thus, under conditions in which classical conditioning between the apomorphine pecking effects and a certain context is not possible, no sensitisation of the pecking response occurs. Consequently, to study the pharmacological processes involved in this drug-induced learning, as well as its anatomical characteristics were relevant. The results presented in this chapter shed some light on this topic. The findings in experiment 3 using binding assay, that pigeons after nine daily apomorphine injections have higher D1/D2 ratios than pigeons injected with saline, indicates that under those conditions, a pharmacological change in the dopaminergic system takes place. With the chronic stimulation of the dopaminergic receptors by apomorphine, D1-like dopamine receptors slightly increased and D2-like receptors slightly decreased. Until now, changes in the pharmacology of this type of learning had remained largely unknown. Whether such changes participate in the mechanism through which learning takes place is a question which deserves further investigation. Moreover, the dopamine participation on synaptic plasticity is a new topic worthy of more attention. Further, the comparison of the outcomes from drug-induced learning research with the possible changes in dopamine receptors emerging after a training with a drug-free learning paradigm seems to be a crucial topic.

The hypothesis that dopamine modulates both LTP induction and maintenance was corroborated by several authors (Centonze et al. 1999, Calabresi et al. 1997, Kusuki

et at. 1997, Frey et al. 1990, Mody et al. 1984). LTP is known to be required for plastic mechanisms necessary for learning and memory (Cain 1997, Rison & Stanton 1995, Teyler & Discenna 1984). Despite that most of the experiments that assessed the involvement of LTP in learning and memory were carried out in hippocampal areas (Cain 1997, Rison & Stanton 1995, for review see Hölscher 1997), some other areas were also investigated. One of these areas was the striatum. Here, the interaction of dopamine and glutamate was demonstrated. Moreover, in the striatum dopamine and glutamate seem to play an interactive role in the sensitisation of a response and in synaptic plasticity (Centonze et al. 1999, Calabresi et al. 1997, Kusuki et at. 1997, Frey et al. 1990, Mody et al. 1984). The modification of dopamine receptor amounts after chronic apomorphine treatment could be consequently interpreted, as a result of synaptic plasticity processes that involve dopamine as well as glutamate receptors. As mentioned before, dopamine stimulation is necessary for induction and maintenance of LTP. Thus, possible changes in dopaminergic receptors could be explained by the action of intracellular mechanisms in the post-synaptic membrane, which could enhance the dopamine receptor synthesis. In fact, NMDA blockade by MK-801 increases the D1- and D2-like receptor amounts in a dose dependent way, in the striatum (Healy & Meador-Woodruff 1996, Kobayashi & Inoue 1993). A cross-gene expression seems to take place, since it was found that apomorphine administration is capable of stimulating the expression of early fos-like genes in the nucleus accumbens, caudado putamen and globus pallidus in rats (Paul et al. 1995). In contrast, the pre-administration of NMDA antagonist prevents the apomorphine effect on the fos-like genes (Paul et al. 1995). Additionally, LTP maintenance seems to be also dependent on fos-like gene expression (Jeffery et al. 1990). Glutamate and dopamine are very closely related in the striatum (Cepeda & Levine 1998, Kalivas 1995). The physiological output of the striatum is a complex interaction between glutamate, dopamine, GABA and acetylcholine systems (Svensson et al 1992a, 1992b). However, these neurotransmitter system interactions remain unclear.

Glutamate also plays an important role in the locomotor stimulatory effects and stereotypes produced by D1-like dopamine receptor stimulation (McPherson & Marshall 1996, Svensson et al. 1994, Svensson et al. 1992a, Carlsson & Carlsson 1990). In addition, the D2-like dopamine receptor stimulation induces stereotypes that are also affected by glutamate (McPherson & Marshall 1996, Svensson et al. 1994). This glutamatergic effect on the dopaminergic activity takes place in the striatum (Cepeda &

Levine 1998, McPherson & Marshall 1996, Kötter 1994). It was found that glutamate stimulation increases the dopamine release (Chaki et al. 1998, Brudzynski & Gibson 1997), as dopamine stimulation also affects the glutamate release in the striatum (Kalivas & Duffy 1997). This very complex relationship between glutamate and dopamine at several levels, such as neurotransmitter modulation and gene expression, is still not fully understood. However, part of the mechanism responsible for the behavioural changes observed in learning seems to occur at the level of the basal ganglia.

Another question concerns the anatomical localisation of the dopaminergic brain areas which are relevant for this type of learning. Godoy and Delius (1999) proposed that, in apomorphine-context conditioning, pigeons learn an association between the unconditioned apomorphine pecking effects US, and a complex conditioned stimulus CS, composed by the context and probably by the effects of apomorphine on perception. Actually, apomorphine readily crosses the blood-brain barrier and in so doing can in principle act on the several dopaminergic systems existing in the brain, and on receptors present in other parts of the body. Dopaminergic receptors have been identified in several tissues, including the retinal cells of different species, among them, pigeons (Glagow & Ewert 1997a, 1997b, Roher & Stell 1995, Djamgoz & Wagner 1992, Schorderet & Nowak 1990). The action of apomorphine on such receptors may alter the internal state and the perception of animals. The fact that systemic apomorphine probably influences several internal parameters of the subjects makes the apomorphine-context association a rather complex paradigm. It is worthwhile to point out that such a complex stimulus (apomorphine effect) has been shown to have a robust capacity to serve as an unconditioned stimulus in a Pavlovian experimental design (Godoy 2000, Godoy & Delius 1999, Wynne & Delius 1995, Kropf et al. 1991, Lindenblatt & Delius 1987).

One possible way to dissect the several components of this type of learning may be the application of apomorphine in localised brain areas. Very little is known about which areas in the pigeons' brain are responsible for the apomorphine-induced pecking behaviour. The crucial areas have not been clearly identified yet. A previous study in our laboratory showed that the nucleus basalis, paleostriatum augmentatum and archiestriatum pars ventralis were capable of inducing pecking behaviour after apomorphine administration (Lindemblatt 1986). Additionally, some inferences can be made from the above cited evidence in other species. However, Experiment 4 through

the implantation of cannuli, provides some information about two brain areas generally found to be involved in locomotion and motor stereotypes, namely the nucleus accumbens and the striatum. The apomorphine administration into the nucleus accumbens showed a significantly higher response than administration into the striatum and control. However, the results described in the present work do not agree completely with those mentioned above. The striatum group consisted of pigeons which had the injection sites in the paleostriatum augmentatum and paleostriatum primitivum. These pigeons did not show any statistical difference from the control group. However, the differences in the experimental design should be taken into account. Lindenblatt injected apomorphine locally into the brain of drug naive pigeons (Lindenblatt 1986). In the present work, the experimental design was made in such a way that pigeons were already apomorphine sensitised. Then animals received local administration of apomorphine to asses the ability of the injected areas to sustain the pecking response. Additionally, the striatum group involved pigeons that had injection sites in the palestriatum primitivum, an area not tested by Lindemblatt. Much more research in this direction is required. Administration of apomorphine simultaneously in both areas as well as it injection in different brain areas are perhaps useful ideas to pursue. Finally, the areas in which dopaminergic drugs induce stereotype behaviours do not have to be necessarily the same as the areas where they induce rewarding effects associated with learning. This is another topic that deserves investigation.

Chapter V:

A novel drug-free associative learning: An association between coloured grit and food

Introduction

The Dutch ethologist Lucas Tinbergen hypothesised that in animals that conduct a visual search for food, a selection bias could result from active processes. He argued that, for a particular type of food, the likelihood of a similar subsequent food discovery should increase, relative to the experience of encountering food of a disparate appearance (Tinbergen 1960). Because the essential feature of this process is a tendency to match or to immediately repeat previous feeding acts, Bond (1983) defined "matching selection" as the bias in favour of the more frequent food types. In Tinbergen's hypothesis "hunting by searching image", the bias is assumed to reflect an improvement in the animal's ability to discriminate food-related stimuli from a background containing features of similar appearance. Cryptic stimuli that are detected more often or more recently are assumed to be more readily discriminated than those with which the animal has had less experience. Other authors have interpreted Tinbergen's perceptual change as an attentional process, conceivably involving both discrimination learning as well as shifts in selective attention among alternative food stimuli (Pietrewicz & Kamil 1979, 1977, Dawkins 1971a, 1971b, Croze 1970).

Tinbergen's hypothesis offers a background for the interpretation of experiments in which animals discriminate among stimuli related to the presence or the absence of food. In a typical stimulus discrimination task, the subjects are exposed to at least two different stimuli, and they are only rewarded for responding to one of them. The stimulus that signals the presence of reinforcement is called the S+ and the other stimulus or stimuli that signal/s the absence of reinforcement is/are called the S-. Discrimination learning experiments may involve different kinds of stimuli, for example, visual stimuli, odour, taste or noise stimuli. The schedule in which the stimuli and the reinforcement are presented may also vary. Thus, in simultaneous discrimination tasks the S+ (leading to reinforcement) and the S- (not leading to reinforcement) are presented to the animal together. In the simultaneous matching to sample procedure, the subjects have learned to associate a certain stimulus among others with a sample simultaneously presented. In the delayed matching to sample procedure, the subject experiences

a stimulus and is later asked to recognise it among different stimuli that are simultaneously presented.

Pigeons are a very good animal model for studying visual discrimination. Among birds, chicks and pigeons have been extensively used as experimental subjects in studies on the avian visual system and on visually guided behaviour. Chicks have a central retinal area of enhanced sensitivity (Ehrlich 1981). Pigeons have two different retinal areas: a small dorso-temporal area called the red field, and the remainder of the retina, called the yellow field. Because of their position, the red fields project in front of and below the beak, and comprise frontal vision fields, which overlap, providing binocular vision. The yellow fields, in turn, project to the sides and provide monocular lateral vision (Jahnke 1984, Martinoya et al. 1981)**.** The end result is acute near vision at short distances and nearly panoramic vision at long ones (Remy & Watanabe 1993). Pigeons are recognised as a laboratory animal with one of the best visual systems. There are numerous examples of experiments using visual discrimination with pigeons (Blough 2000, Gargiulo et al. 1998, Wright & Delius 1994, Hahmann & Güntürkün 1992, Wright 1992).

Simultaneous visual discrimination is a useful tool to study learning and memory processes. Along these lines, different types of visual discrimination experiments have been used to study learning in pigeons: Wright (1992) has trained pigeons to associate a certain stimulus to food with a simultaneous matching to sample procedure. Gargiulo et al. (1998) studied the simultaneous discrimination of a certain rewarded stimulus. The transfer of the S+ value has been studied in transitive inference experiments by von Fersen et al. (1991). Similarly, this task may be used to study memory processes, assessing whether pigeons remember visual shapes, certain responses, the presence or absence of reward, and the spatial location of stimuli. Simultaneous discrimination may also be used in conjunction with other techniques such as lesions of certain areas in the central nervous system, or the administration of drugs that affect learning (Harder et al. 1998) or visual processes. Discrimination procedures also allow, for examining axon regeneration after lesions (Thanos et al. 1997) or recording neuronal activity from different brain areas during learning (Wang & Dreher 1996, Gochin et al. 1994).

Simultaneous discrimination of a visual stimulus

Experiment 5. The purpose of the following experiment was to design a quick and reliable learning paradigm for pigeons which could later be used as a tool to assess different factors which could influence the associative learning process. It is well known that natural behaviours

are easy to adapt to learning experiment procedures. During foraging, pigeons occasionally have to remove leaves, twigs, or gravel. Thus, the natural "digging" with the beak when searching for food, could in principle be adapted to an associative learning paradigm. A task was developed in which pigeons were confronted with several pots filled with grit of different colours, only one of them containing buried food grains at the bottom. Pigeons were expected to spontaneously dig in the grit pots to search for food. This design was created in an attempt to assess whether pigeons would learn an association between the specific coloured grit containing food and the reward, and use it as a discriminative stimulus to perform the digging response. By making grit, the material covering the food reward, the discriminative stimulus problems like colour preferences associated with using the grain itself as the discriminative stimulus (Siemann & Delius 1992a, Jäger 1990, Güntürkün & Kesch 1987, Bond 1983) should be avoided.

The development of such a task was based on an earlier matching-to-sample paradigm designed by Wright and Delius (1994). These authors pre-trained pigeons to dig in a pot filled with grit until they learned to scatter the grit out of the pot and in so doing to get the buried grain. The training consisted then in a matching-to-sample procedure in which a plastic tray containing three grit-filled pots was presented. Seeds were buried at the bottom of the central sample-pot. When the pigeon was eating the second seed, the comparison-pot covers were opened and pigeons had to chose one side to dig. The correct side pot contained seeds buried at the bottom and the incorrect was seed-free. In this chapter, a description of a simultaneous visual discrimination of a reward-associated pot, filled with coloured grit among several others simultaneously presented is done. This task will be then used to evaluate the influence of glutamatergic and dopaminergic drugs on learning, described in the following chapters.

Method

The cage. The experimental cage was a standard grid cage, normally used to home pigeons, with the lateral sides covered with white cardboard sheets and the back side up against the wall of the experimental room. In order to reduce disturbances during the task, subjects were thus visually isolated from each other to a certain degree. Six identical plastic pots, 83 mm wide x 43 mm deep x 54 mm high, hung inside the cage at 91 mm height (figure 5.1b). The different coloured grit of pots consisted in the small coloured stones, typically used in aquariums, with a similar shape and a diameter (approximately 2,5 to 3,5 mm). Every pot was filled with grit of a

different colour: black, red, blue, yellow, green and light grey. Two of the six pots were placed on the front of the cage and two on each side. The position of the different colours could be varied during the experiment. Figure 5.1a shows a scheme of the experimental set-up with an example of colour distribution. The pots without food (S-) contained only coloured grit to a depth of 8 – 10 mm. In the pots containing food (S+), 5g of millet seeds were distributed in the bottom and covered with the same amount of coloured grit.

Procedure. Experiment 5 consisted of two phases.

First phase. In the first phase, two groups of 11 mildly deprived pigeons each, were used. Group A underwent four discrimination training sessions. For these sessions, one coloured grit was designated as the positive stimulus (S1+) while five other stimuli were non-reinforced (S-). To avoid any spontaneous colour preference bias, every second pigeon of this group was trained with a different colour as the S1+. Group A was called in the first phase D(S1+). Thus, for two pigeons of group D(S1+), the blue grit was the S1+, and for two other pigeons of the same group, the yellow grit was the S1+, etc. Since the group consisted of 11 pigeons, only one experienced the colour red as the S1+. Millet was placed at the bottom of one of the pots in the cage and subsequently covered with the S1+ coloured grit. A shaping procedure was used. To facilitate the initial response, 4-5 millet seeds were put on top of the S1+ grit, for the first two training sessions. In the last two training sessions, the millet seeds were not visible. Two test sessions followed the training. Here, all the grit pots were presented without food. Pigeons responses to the different colours were assessed.

Group nD also underwent four training sessions, all the six pots containing millet at the bottom. During the first two training sessions, these pigeons were also shaped with a few seeds placed on the top of each grit-containing pot. Thus, group nD received a non-differential training since no colour was associated with food and all the colours were equally rewarded. Because no particular stimulus signals a reward, this kind of training will be here referred to as S^0 training. Consequently, this group was called nD(S^0). After training, two foodless test sessions were

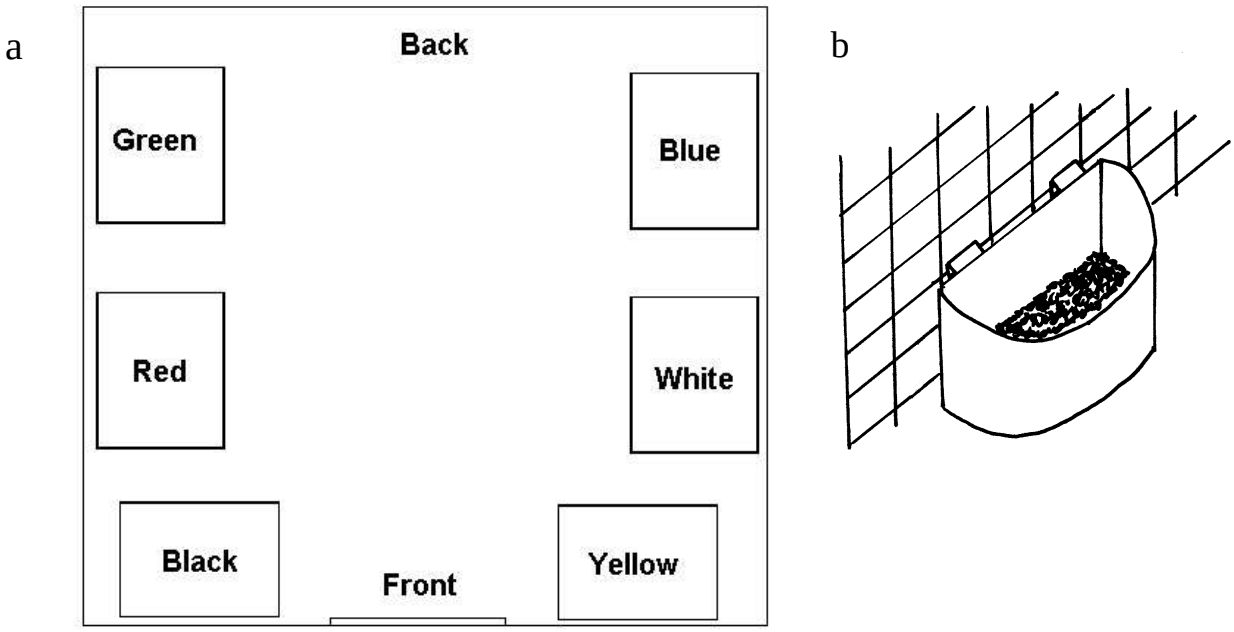


Figure 5.1 a) Schematic representation of the arrangement of the six grit-containing pots inside the experimental cage, with an example of the distribution of colours. The position of each colour varied in a quasi random order from session to session, so that one colour never occupies the same place. **b)** Schematic detail of a pot containing 1 cm deep layer of grit**.**

carried out. Training sessions lasted for 20 minutes while test sessions were 10 minutes long. Sessions were carried out twice per day, once in the morning and once in the afternoon, with an inter-sessions interval of 6 to 7 hours. Pigeons were video-recorded so that their responses could be assessed later. The positions of the pots with different coloured grits were varied in a quasi- random procedure from session to session. This procedure was performed such that a specific colour never occupied the same place.

Second phase. The second phase of this experiment was designed to assess to what degree a discriminative training (group D trained with an S1+ versus several S-) and a non-discriminative training (group nD trained with S^0) procedure could influence the subsequent learning of a new discrimination with a different positive stimulus, S2+. In other words, to assess the hypothesis that this simultaneous discrimination paradigm may undergo a partial-reversal learning procedure. Additionally, a second hypothesis was whether this learning paradigm could be subject to a latent inhibition procedure. Group A was re-trained (four sessions) using a different colour as the positive stimulus, S2+, while the others were the non reinforced S-, a partial reversal learning procedure. Group D was then called D(S2+). Group nD was re-trained (four sessions) using one certain coloured-grit as the positive stimulus, called

S+, while the rest of them were the non reinforced S-, a latent inhibition procedure. Group nD was then called nD(S+). For these re-training procedures, no shaping procedure was used, because all pigeons had already been used for the type of response required. Two test foodless sessions followed the retraining.

Analysis. For the analysis of pigeon performance in this experiment, two parameters were taken into account. The *preference***,** indicated by the number of times that a pigeon chose to peck on a certain stimulus, i.e. the "number of visits" to that stimulus, here referred to as **V**. A visit was counted every time a pigeon approached and pecked one time on a certain pot, independent of the subsequent number of pecks on the same pot before it withdrew. If the pigeon then went away or stopped pecking and returned later, or started to peck again on the same pot, its first new peck was again counted as a visit. The *strength* or value of the stimulus was indicated by the total "number of pecks" on it during a session, here referred to as **P**. A pecking behaviour was considered a bout of pecking when a train of continued pecking with inter-peck intervals of less than 2 seconds occurred.

To compare preference and value strength among the different stimuli, the percentage of visits and pecks to each one was calculated. The percentage of visits to a certain stimulus S+ was

$$(V_{S+} / V_T) * 100$$

where V_{S+} was the number of visits to a stimulus S+ during a session, and V_T was the total number of visits to all the stimuli in that session. Similarly, the percentage of pecks to a certain S+ was

$$(P_{S+} / P_T) * 100$$

where P_{S+} was the number of pecks on an S+ during a session, and P_T was the total number of pecks to all the stimuli during that session.

However, these percentages did not show the expected results. Several interpretation problems arose when the percentage of visit and pecks to the S+ were used. The percentage of pecks seems to be a parameter not sensitive enough, since after the 2nd session it almost always reaches the top of the scale (98%-100%). In contrast, the percentage of visits to a given stimulus yields highly variable results. Additionally, the percentage of visits and pecks may lead to somewhat contradictory interpretations. Pigeons may frequently visit very few times a certain coloured grit pot, but pecking in it many times. In this case, the percentage of visits may reflect a low preference for that colour, while the percentage of pecks indicates that this colour

has a high value for the pigeon. To avoid such contradictions, and in order to describe the response of pigeons to a given stimulus in a more complete and reliable way, a *discrimination index, DI,* using both variables is proposed. Visits and pecks are combined as follows

Discrimination Index = Preference for a certain stimulus * Strength value of that stimulus

which, referring to a certain stimulus S+, would be expressed as

$$DI_{S+} = (VS+ / VT) * (PS+ / PT)$$

As already mentioned, V_{S+} is the number of visits to the S+ pot during a session, V_T is the total number of visits to all the pots in that session, P_{S+} is the number of pecks on the S+ stimulus during the session, and P_T is the total number of pecks to all of the stimuli. This index ranges between 0 and 1, with 1 representing the maximum discrimination of a certain reward-associated stimulus and 0 representing no discrimination. The index might be thought of as the weighted value of the choice to a given stimulus. A correlation analysis between the percentage of pecks to a certain S+ and the DI and the correlation between the percentage of visits to the S+ and the DI yielded statistically significant correlation coefficients: ($r^2 = 0.77$, $r^2 = 0.97$, with a $p \leq 0.05$ for pecks and visits respectively). During the first phase, group nD(S^0) was trained with a non-discriminative procedure. Accordingly, it responded at a chance level for all stimuli and had the lowest index values of both groups. These values can be used as a baseline for comparisons with other results.

Inter-group comparisons were done using Student t-tests or ANOVAs for independent samples. Intra-group comparisons were done with Student t-tests or ANOVAs for dependent samples.

Results

First phase. One pigeon of group nD(S^0) never approached the pots and showed only minimal exploration during training and test, and was therefore discarded. The remaining pigeons in this group showed consistent exploration and a total number of pecks per session comparable to that of group D(S1+). Figure 5.2 illustrates the response of group D(S1+), trained to discriminate a

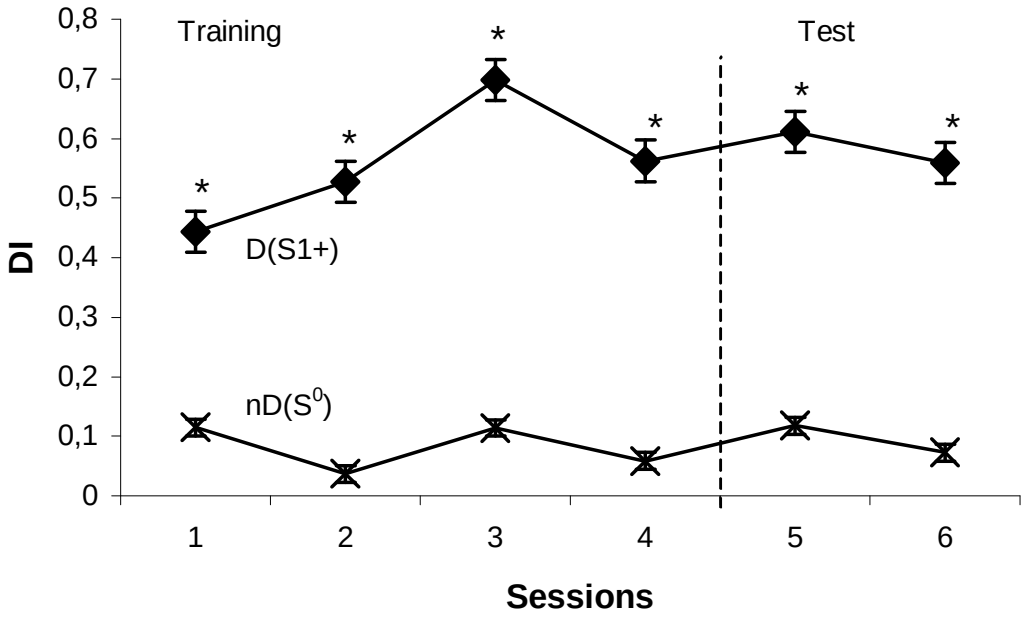


Figure 5.2. Averaged DI values ± SE of groups D(S1+) and nD(S^0) during the first phase (training sessions 1 to 4; and test sessions 5 and 6) of experiment 5. Curve S1+ and curve S^0 show significant differences between them for every session (* indicates $p \leq 0.05$).

given coloured grit (S1+) among six pots, and group nD(S^0), trained with a non-discriminative procedure, in which all the stimuli were equally rewarded, as described by the discrimination index, DI, averaged for all pigeons in each group. As expected, group nD(S^0) responded at a chance level. In this experiment, the chance level was 1/6, or 16.6% of the visits and pecks, which corresponds to a DI equal to 0.028. The DI values for group nD(S^0) were calculated at the highest values from each pigeon. This arbitrary decision was adopted in order to have the highest baseline possible. In so doing, a robust result conclusion could be made. Statistical differences were found for every session of training and test when the DI values of groups D(S1+) and nD(S^0) were compared ($p \leq 0.05$). These differences suggest that group D(S1+) learned to discriminate a positive stimulus independently of the stimulus colour. Group D(S1+) shows high DI values and this from the beginning of training, increasing until the 3rd session. These high DI values were maintained during the tests.

Figure 5.3 shows the DI values of group nD(S^0) for each coloured grit. In contrast to figure 5.2, the value scale in this graph has the maximum ID value of 0.1 (the range of the ID value is from 0 to 1). These results suggest some colour preferences. In fact, the ID values for

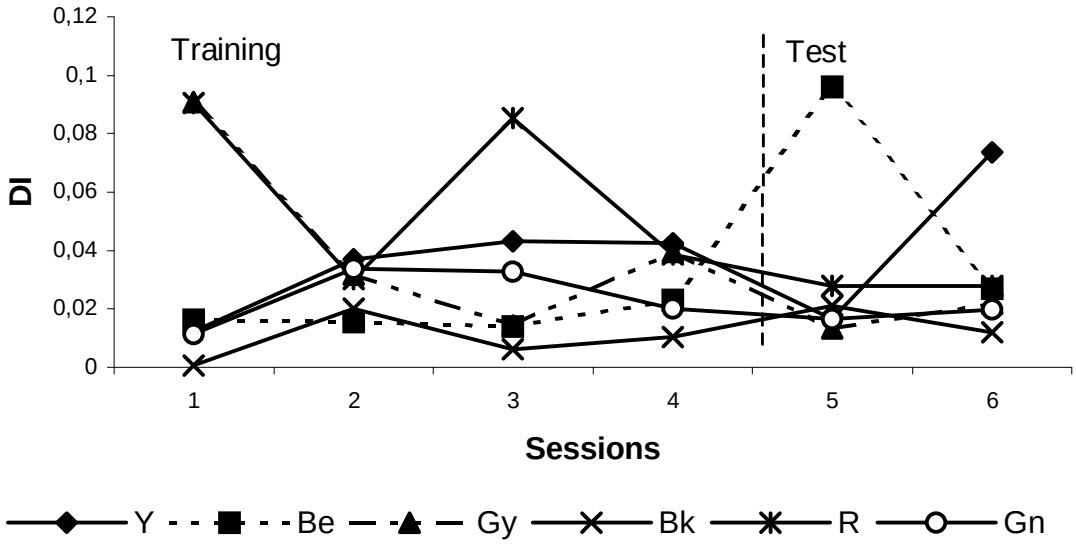


Figure 5.3. Averaged DI values of group nD(S^0) for each coloured grit. These results suggest a preference for red and grey during the training and for blue and yellow during the test. A multivariate Anova yielded statistical differences among colours ($p \leq 0.05$). Y = Yellow, Be = Blue, Gy = Grey, Bk = Black, R = Red and Gn = Green.

the red and the grey coloured grits are higher than the others during training, while the ID values for the blue and the yellow grits are higher than the others during test. A multivariate ANOVA test showed that the ID values in the nD(S^0) group were statistically different, depending on grit colours. ($F_{5,5} = 2.62$; $p \leq 0.05$).

Second phase. During the second phase of the experiment, group A was trained to discriminate a second positive stimulus S2+ (after being trained to discriminate S1+ as the positive stimulus in the first phase). So, the group D was then called D(S2+). Additionally, group nD was trained to discriminate a positive stimulus S+ for the first time (after a non discriminative training in the first phase). This group was called nD(S+). Five pigeons of group D(S2+) had to be discarded from this second phase, because they made no pot visits during the four sessions of training. The 10 pigeons used in group nD(S+) during the first phase continued to work well.

Figure 5.4a shows the DI values for both groups in the second phase of experiment 5. No statistical difference was found between D(S2+) and nD(S+) groups. For comparison, figure 5.4b shows the results of the second phase (curves D(S2+) and nD(S+)) together with those of

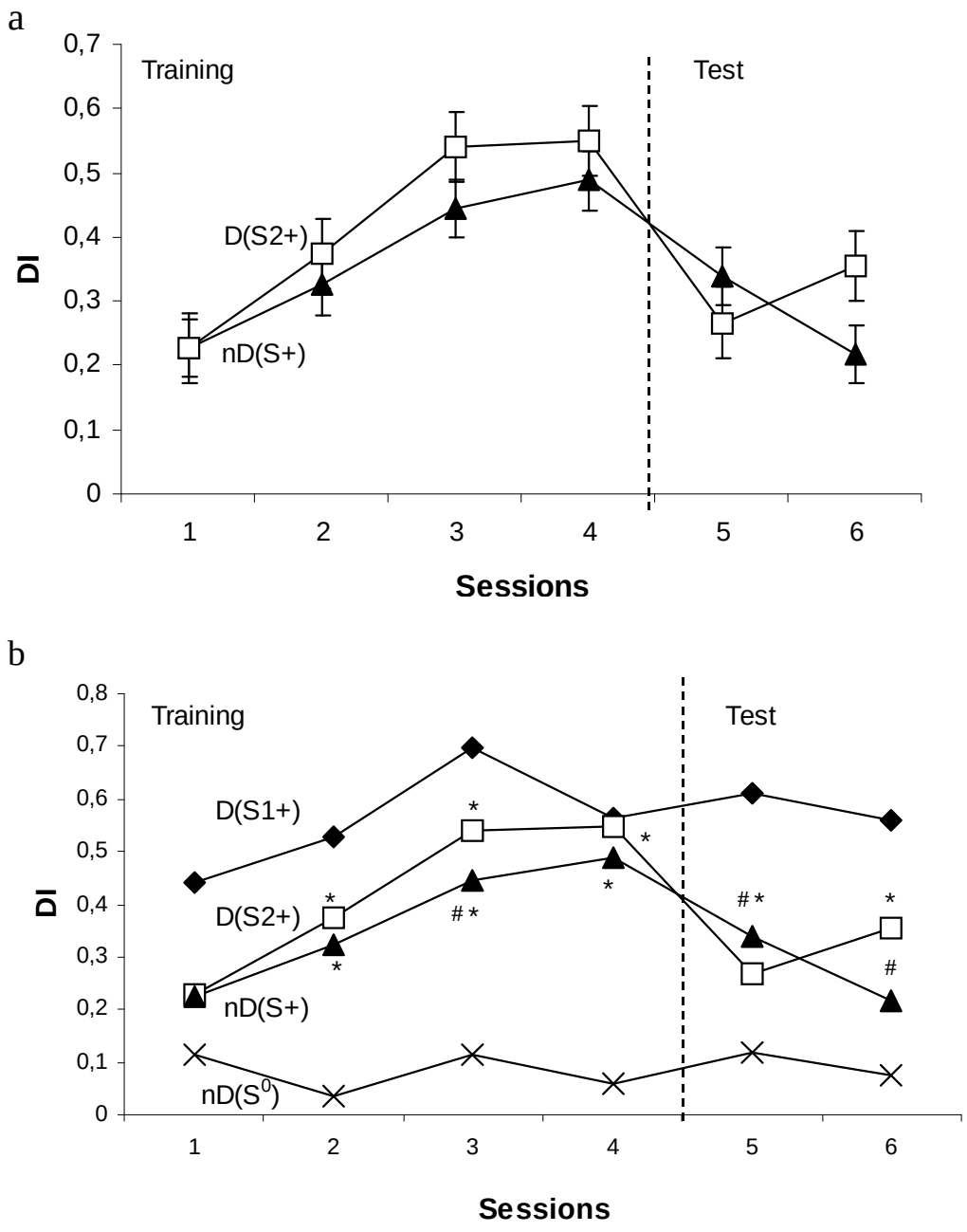


Figure 5.4. a) Averaged DI values ± SE of D(S2+) and nD(S+) groups during the second phase of experiment 5. There were no statistical differences between them. **b)** Averaged DI values of D and nD groups during the first and the second phase of experiment 5. Curves D(S1+) and D(S2+) correspond to the first and the second phase of D group respectively. Curves nD(S^0) and nD(S+) correspond to the first and second phases of nD group respectively. (*) indicate statistically significant differences ($p \leq 0.05$) of the curves as compared with nD(S^0), the chance level (see main text). (#) indicates statistically significant differences ($p \leq 0.05$) of the curves as compared with D(S1+), a first-hand discriminative learning task; see main text.

the first phase (curve D(S1+) and curve nD(S^0)). As can be observed, the general performance of group D(S2+) was lower than its performance during the first phase, D(S1+). This result suggests that pigeons found it more difficult to learn a second discrimination task after they had learned a different task in the same situation. However, no statistical differences were found when the D(S1+) and D(S2+) were compared. Statistical differences were found for sessions two to four and six, when curve D(S2+) was compared with the baseline nD(S^0) (Student t-test for non-paired samples, $t_{15} = 6.68$, $t_{15} = 4.57$, $t_{15} = 5.32$, $t_{15} = 3.50$ for 2nd to 4th and 6th sessions respectively; $p \leq 0.05$). This indicates that pigeons effectively learned the association of D(S2+) with food, even when this new learning seemed to be worse than the first discrimination with D(S1+). Figure 5.4b also shows the response of group nD(S+) during the second phase of the experiment. Responses here were significantly higher than responses during the first phase nD(S^0) (Student t-test for paired samples, $t_9 = 4.39$, $t_9 = 3.26$, $t_9 = 6.84$, $t_9 = 2.76$ for 2nd to the 5th sessions; $p \leq 0.05$). In phase one, this group responded at the chance level, which can be interpreted as reflecting the newly acquired discrimination learning.

At a first glance, curves D(S2+) and nD(S+) show that both groups responded at a similar level during the second phase. This suggests that a non discriminative strategy and a previous learning with a different discriminative stimulus similarly affect a subsequent discrimination. However, despite this apparent similarity, differences arise when these curves are compared with curve D(S1+), a first-hand discriminative learning. The difference between D(S2+) and D(S1+) was not significant in any session. On the other hand, curve nD(S+) was statistically lower than D(S1+) in the 3rd session of training and in both test sessions (Student t-test for non-paired samples, $t_{19} = 2.13$, $t_{19} = 2.47$, $t_{19} = 3.23$ for 3rd, 5th and 6th sessions respectively; $p \leq 0.05$). During the second phase, both groups showed a decrement of the DI values during test. However, only nD(S+) showed statistically significant decrement when compared with D(S1+). Additionally, the first and last DI value of curve nD(S+) did not differ significantly from the respective values of the nD(S^0) curve. These two later outcomes suggest that the discrimination abilities of pigeons after a non-discriminative procedure, nD(S+), were effectively worse than those of pigeons learning to discriminate without such a previous procedure, D(S1+). Nevertheless the 5th session of the D(S2+) was not significantly higher than the baseline nD(S^0).

Even when the tendency is slight, the above described differences between D(S2+) and nD(S+) are not surprising. It can be speculated that the effects of previous experience on subsequent learning, when the same stimuli and experimental situation are involved, are more

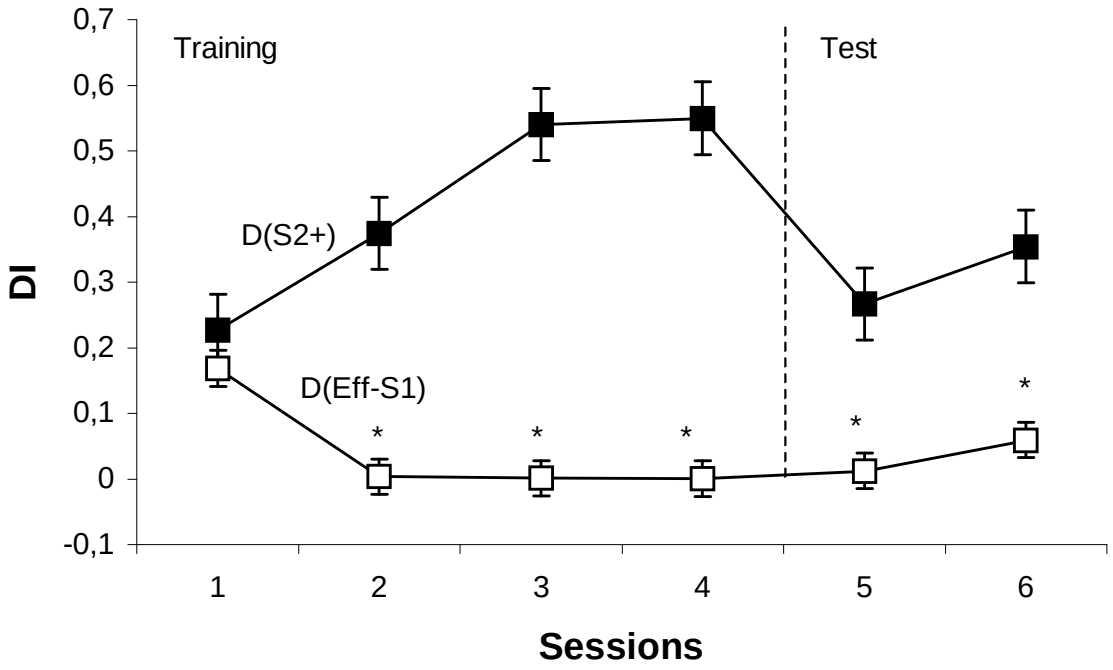


Figure 5.5. Average of DI values ± SE of group D(S2+) and D(Eff-S1) of experiment 5, second phase. The effects of a previous discriminative training, with a particular positive stimulus, D(Eff-S1), on the subsequent discriminative training with a new different positive stimulus, D(S2+). Statistical differences between groups were found in every session excepting the first one (* indicates $p \leq 0.05$).

disruptive, compared to a situation involving different stimuli and experience. Thus, D group during the first training would have learned to discriminate the corresponding positive stimulus among the others and additionally learned something about the required task. For the second phase, these pigeons already knew what they were required to do, even when they had to re-learn the appropriate positive stimulus. Conversely, group nD was not required to make a discrimination during the first phase. These pigeons had to learn the positive stimulus and the type of task, for the first time, during the second phase.

To evaluate whether pigeons in group D(S2+) during training with S2+ as the positive stimulus would still prefer S1+ (the first learned positive stimulus), the following analysis was performed. Figure 5.5 shows the DI values of group D(S2+) during the second phase for the now positive coloured grit and for the former positive coloured grit during also the second phase (curve D(eff-S1)). Thus, the D(eff-S1) are the DI values for the previous S1+ that pigeons performed during the second phase. It is important to point out that in the second phase, the previous S1+ had no more food, being therefore a negative stimulus (S-). The curve

D(eff-S1) indicates to what degree S1 (the previous S1+) is still preferred during the training to S2+. No difference can be appreciated in the first training session, suggesting that pigeons respond to S1 as strongly as to S2+. The fact that both responses are significantly higher ($p \leq 0.05$) than the first training session of the chance level curve, nD(S^0) (compare figure 5.2 with 5.5) suggests that pigeons were not randomly responding to all stimuli, including S1+ and S2+. It is tempting to speculate that the new learning of a positive stimulus is in conflict with the still preferential response to the former positive one, yielding an overall similar result for both. With some additional training, i.e. starting in the second session, pigeons clearly differentiate the new positive stimulus. They respond significantly more to it than to the former one (Student t-test for paired samples, $t_5 = 5.35$, $t_5 = 5.30$, $t_5 = 4.38$, $t_5 = 3.02$, $t_5 = 2.60$ for 2nd to 6th sessions respectively; $p \leq 0.05$). Actually, they practically stop responding to S1+. As expected, the DI values for S1 during the second phase were statistically lower than chance level, nD(S^0) in the 2nd and 4th sessions (Student t-test for non-paired samples, $t_{15} = 3.49$, $t_{15} = 2.99$ for 2nd and 4 sessions respectively; $p \leq 0.05$).

Discussion

First phase. The results obtained in the first phase of experiment 5 indicate that pigeons readily learn to associate food with a stimulus (such as a coloured grit) they have to dig in. The coloured grit presented consistently together with food, came to serve as the discriminative stimulus for pigeons in group D(S1+). As expected, pigeons in group nD(S^0), which experienced all the stimuli associated with food, did not learn to discriminate any of them from the others. Despite some spontaneous colour preferences, pigeons did not show any discrimination among colours associated with food. This spontaneous colour preferences for blue and yellow, in the nD(S^0) group, were congruent with previous results. Using an operant technique, it was demonstrated that blue and yellow-green colours were spontaneously preferred by hungry and thirsty pigeons (Sahgal & Iversen 1975, Delius 1968). To avoid this spontaneous colour preference in the discriminative training, treatment consisted of different colours as the positive stimulus (S+) for different animals. The D(S1+) group showed an increase of the DI values until the third session, then these values stayed almost constant during the last training and test sessions. As mentioned before, the main conclusion of this experiment is that pigeons learned to discriminate a given stimulus independently of the stimulus colour. This learning paradigm shows a clearly differentiated response between the discriminative and

the control groups. This is absolutely necessary when used to perform pharmacological tests to study the possible neurotransmitter systems involved in such learning processes.

Second phase. The results obtained during the second phase of this experiment indicate that both a previous different discriminative procedure and a previous non-discriminative procedure, impaired the subsequent discriminative training. In classical conditioning, subjects are repeatedly exposed to a certain, in principle irrelevant, stimulus (conditioned stimulus, CS) consistently paired with another stimulus which does have an effect on the subject (unconditioned stimulus, US) and spontaneously induces a response (unconditioned response, UR). After several paired presentations of both stimuli, the first, irrelevant CS starts to induce a response (conditioned response, CR), which is normally very similar to the UR. The subjects learn to associate both stimuli and respond to the former irrelevant one as if they were confronted with the UR. It is well known that this kind of associative learning can be impaired by a procedure called CS pre-exposure or latent inhibition.

Lubow & Moore (1959) defined latent inhibition as a decrement in the learning performance resulting from the non-reinforced pre-exposure to the to-be-conditioned stimulus. In other words, when animals have experience with a certain stimulus without any special association, and that stimulus is later used as the CS in a classical conditioning procedure involving a US, the learning of the US-CS association is slower than in animals for which the CS is a novel experience (Domjan 1993). In discrimination learning, the previous exposure to a certain stimulus has also been found to impair the subsequent acquisition of that particular stimulus as the one to be discriminated (Schauz & Koch 1998, Killcross & Balleine 1996, Kilcross & Dickinson 1996, Lubow 1989, and for review see Hall 1980). It seems to be the case that the pre-exposure to the stimuli has some effect on the distinctiveness of that stimuli (Hall 1980).

In experiment 5, group nD(S^0) was exposed to all of the coloured grit stimuli associated with the same reward, during the first training, in a non-discriminative procedure. During such a procedure, pigeons can be thought to have acquired a kind of "learned irrelevance" with regard to those stimuli. During the second training, the pigeons were required to associate particularly one of those stimuli with the reward. As expected, these pigeons learned to discriminate a particular S+ in the second training (comparison between nD(S^0) and nD(S+) in figure 5.4b), although their performance was lower than that of pigeons, which learned such a discrimination for the first time (comparison between nD(S+) and D(S1+) in figure 5.4b). The

DI values of nD(S+) group during test, were statistically lower than D(S1+) group, a first hand discriminative learning. This results support the hypothesis that pre-exposure of the discriminanda stimuli impairs the subsequent acquisition of a discriminative learning.

Another phenomenon that influences learning is so-called reversal learning. In a reversal learning procedure, subjects first learn that a certain stimulus X brings a reward, i.e. it is a positive stimulus, while any other stimuli are negative or non-rewarded, for example a non rewarded stimulus Y will be the negative stimulus. In a subsequent phase, the allocation of the reward is changed so that the former positive stimulus is no longer associated to the reward, becoming a negative stimulus At the same time, a former negative stimulus is now associated with the reward and in so doing becomes positive. It is a consistent general finding that subjects perform better during the initial training when they learn a first stimulus X-reward, stimulus Y-no reward association, than in a subsequent training, when they have to "disregard" what they have learned and acquire an inverse stimulus X-no reward, stimulus Y-reward association. Reversal learning has been studied in a variety of species including rats (Nakagawa 1992) and pigeons (Delius et al. 1995) with the consistent result that subjects either show a worse performance with the reversed schedule of reinforcement than with the original one, for the same amount of training, or need more training with the reversed schedule than with the original, in order to achieve the same performance level.

In experiment 5, group A underwent a first training, where a certain coloured grit S1+ was the rewarded stimulus, while all the other ones were negative stimuli (not rewarded). During the second training, the formerly positive S1+ coloured grit was no longer rewarded with food. Thus, it became a negative stimulus. Additionally, another coloured grit, not rewarded in the first training (i.e. was negative), became the positive one S2+ in the second training. Pigeons in this group can be considered to have undergone a reversal procedure with regard to the particular S1 and S2 coloured grits. As expected, the results reflect a lower performance of D(S2+) group during the second than during the first training (D(S1+) group), although the differences were not statistically significant. Nevertheless, the preference for the new S2+ or the old S1+ positive stimuli during the first session of the second training, were at the same level. However, they immediately became different one another after the second training session. These results support the hypothesis that a partial reversal learning process impairs the acquisition of the new learning, when the stimuli involved in the association reverse their values. Moreover, this impairment is stimulus specific, since only the previous S1+ was

capable of impairing the acquisition of the new association (S2 with food) and not the remaining four negative stimuli.

One can speculate about the theoretical explanations for the different effects that have partial reversal and latent inhibition procedures on a successive discrimination tasks. As mentioned before, the previous training with a certain discriminative stimulus seems to have less a disruptive effect on a subsequent discrimination with another stimulus, than a previous training, in which no discrimination can be learnt. This probably reflects the fact that pigeons learn something about the type of task, besides learning about the specific stimulus they have to discriminate. This finding is not surprising. It has been repeatedly reported that experimental subjects that have previous experience with a certain task perform better than subjects that face it for the first time, even when the experimental set-up may have changed (Slotnick et al. 2000, Mackintosh et al. 1985). Learning experiments, where different stimuli groups are used consecutively for the same procedure but with the stimuli changed each time, are a called learning-set. Efficient learning-set performance requires the subject to transfer the learned rule across changes of stimuli (Mackintosh et al. 1985). However, not all vertebrates are equally adept at such a transfer. For example, it was demonstrated that corvids exhibit significantly better transfer than pigeons (Lazareva et al. 2001, Mackintosh et al. 1985, Kamill & Hunter III, 1970). Most of these learning-set experiments were performed with a discrimination procedure. In the present work, pigeons did not perform better in the second learning procedure than in the first one. But the effect of the previous acquisition of a discrimination task disrupted the acquisition of the following one, with the same set of stimuli. Learning-set procedures use a large number of stimuli sets. A general versus specific rule learning could have taken place in the partial reversal learning procedure of the simultaneous discrimination. Long ago, Harlow (1959), observed that animals, after a learning-set procedure, had exceptional levels of discrimination between novel stimuli. He and other authors thought that these levels of discrimination could only be achieved if subjects adopt a response "rule" or strategy like "win-stay, lose-shift". In a discrimination task, the rule "win-stay, lose-shift", could occur simultaneously with the conditioned association of "colour-food". In experiment 5, when the conditioned association changed, but, the general procedure was kept valid (for example in the case of D(S1+) → D(S2+)), the impairment of the acquisition of a new discrimination was not so pronounced as when the general procedure was also changed (e.g. in the case of nD(S^0) → nD(S+)). This general procedure was the presence of food in only one pot/colour. This allowed

animals to perform the strategy “win-stay, lose-shift”. Moreover, one can speculate that after several executions of the same procedure, the level of discrimination increases. Despite of the similarities between the partial reversal learning and the learning-set procedures, the performance of pigeons in the partial reversal procedure D(S2+) much worse than in the first hand discrimination task D(S1+). The reversal and the learning-set procedure have not been fully studied yet. This experiment opens a wide field of possibilities to study such kind of learning.

To conclude, the results of experiment 5 indicate that the discrimination of a certain reward associated coloured grit among others which are not reward-associated is a consistent and reliable learning paradigm. This discrimination task could also serve to study other learning associated processes, for example, latent inhibition, extinction, and how the discrimination could be modulated by the value of the stimuli. In this work, this paradigm will be used in further experiments as a tool for assessing the effects of different dopaminergic and glutamatergic drugs on learning. Additionally, the effect of such treatment on this drug-free form of learning can be compared with the effect on a drug-induced form of learning, namely the apomorphine-context association described in chapter III.

Chapter VI

Role of dopamine and glutamate during the grit-food association

Introduction

In the last years, many studies focused their interest on the role of the dopaminergic and glutamatergic neurotransmissions in associative learning paradigms (Fagnou & Tuchek 1995, Morris & Davis 1994, Beninger 1993). Visual discrimination is one of the most frequently used procedures to carry out these pharmacological studies on learning. Visual discrimination has been used in the common marmoset to test for the blocking effects of the dopaminergic D2/D3 receptor agonist 7-OH-DPAT (Smith et al. 1999). Using a discrimination task, several drugs (dopaminergic, serotonergic, GABAergic and glutamatergic agonist and/or antagonists) were tested in animals. Pharmacological stimulation of serotonin receptors (5-HT_{1A} and 5-HT_2) improved the discrimination performance, whereas blocking 5-HT_2 impaired it Evenden 1999b). Haloperidol and chlordiazepoxide improved the performance of a discrimination task in rats (Evenden 1999a). In pigeons, the administration of the anxiolytic diazepam, a GABA-A receptor agonist, abolishes spontaneous colour preferences (Sahgal & Iversen 1975). Also in the marmoset, the NMDA antagonist MK-801 was found to impair the acquisition of a visual-spatial task involving coloured objects (Harder et al. 1998). Similarly, as for mammals, glutamatergic antagonists administrated directly into the nucleus accumbens septi of pigeons blocked the visual discrimination of shapes, while the dopaminergic agonist apomorphine did not affect it (Gargiulo et al. 1998). In chicks, glutamatergic antagonists impair visual discrimination tasks (Tiunova et al. 1996).

The importance of dopamine and glutamate activity in learning and memory processes has already been described. It is however worthy to remark that dopamine plays a central role in the control of locomotor activity and in reward-related incentive learning. On the other hand, the activation of glutamatergic transmission is a crucial step in the induction of long-term potentiation (LTP). LTP is one of the central types of synaptic plasticity assumed to take part in learning and memory processes (Morris et al. 1990). According to the learning model developed by Wickens (1990), there is a relationship between the dopaminergic and the glutamatergic pathways in the striatum, which would be related in a post-synaptic way, probably setting the basis for learning.

The idea behind the experiments presented in this chapter is to examine the role of dopamine and glutamate in associative learning, by assessing the effects of dopaminergic and glutamatergic antagonists on drug-free learning. The simultaneous discrimination of food-associated coloured grit described in chapter IV was used. For the assessment of their effects on the acquisition and the retrieval processes, three experiments were carried out, each of them using a different receptor antagonist administrated either before training or before test sessions.

The receptor antagonists used in these experiments were: 1) **haloperidol** (a selective D2-like receptor antagonist), 2) **SCH-23390** (a potent and selective D1-like receptor antagonist), and 3) **MK-801** (a non-competitive NMDA glutamatergic receptor antagonist). Haloperidol is a butyrophenone frequently used in clinics for its neuroleptic effects. This drug has high affinity for D2-like receptors. At the same time, it also has low affinity for the 5-HT_2 serotonin receptors and a still lower affinity for D1-like receptors (Hacksell et al. 1995). At the concentrations used in clinics, haloperidol only binds to the D2-like dopamine receptors.

SCH-23390 is a benzazepine known to be highly specific for D1-like receptors. Nevertheless, at very high concentrations, it also shows some binding to 5-HT1c and 5-HT2 serotonin receptors (Billard et al. 1984). SCH-23390 has a relatively short half-life in primates (Barnett et al. 1986) and therefore cannot be regularly used as an antispychotic. Despite its short half-life, it has been found to impair a wide variety of typical behavioural paradigms. For example, it inhibits stereotypy, hyper-locomotion, spontaneous activity, and climbing, and induces catalepsy, among other effects.

The experimental evidence obtained during the last 15 years has confirmed that D1 and D2 interactions are a characteristic of many behavioural processes (Beninger 1993; Cooper & Al-Naser 1993). These studies indicate that the level of D1 activity appears to exert important qualitative and quantitative co-operative/synergistic control of D2-stimulated motor activity allowing the full expression of dopamine-dependent behaviours (Waddington & Daly 1993; Zarrindast & Amin 1992). Similarly, in the reverse situation, the D2 activity seems to modulate the D1-stimulated motor behaviours (Waddington & Daly 1993).

The dibenzocycloalkenimine (+)MK-801, is a potent non-competitive NMDA-receptor antagonist. A particularity of this drug is its capacity to block the NMDA-receptor associated channels but not the glutamate binding site. MK-801 acts at the phencyclidine site (PCP) of channels associated with NMDA receptors, selectively reducing their excitatory action. Since the PCP site is located very deep in channels associated with NMDA receptors, MK-801

binds deep within the channel, blocking the normal ionic flux (influx of Ca^{2+} and Na^{+} and efflux of K^{+}).

As mentioned before, three experiments presented in this chapter were designed to test the influence of the dopaminergic and glutamatergic antagonists on drug-independent learning. This was done using the simultaneous discrimination of a rewarded coloured grit. The paradigm was fully described in chapter V. Again, it consists of i.m. injecting mildly deprived pigeons with the corresponding drug solution and immediately placing them into the experimental cage, where their behaviour is video recorded. Training sessions last for 20 and test sessions for 10 minutes. Two experimental sessions per day were carried out. The experimental cage (Showed in chapter V, figure 5.1) contained six plastic pots each filled with grit of a different colour. The colour could be rotated so that each colour had a different location in every session. Only one coloured grit served as the positive stimulus (S+), associated with food reward. This pot contained 5 gr. of millet seeds buried at the bottom. As explained in experiment 5, the recorded variables were the proportion of visits (number of visits to a certain pot relative to the total number of visits to all the pots), and the proportion of pecks (number of pecks elicited on a certain pot relative to the total number of pecks in the session). These proportions were used to calculate a discrimination index (DI), which describes the pigeons' performance. Higher DI-values indicate good discrimination. The indices were analysed by means of parametric tests, two-factorial ANOVAs and Student t-test for dependent or independent samples.

Effects of haloperidol on the coloured grit discrimination

Experiment 6.

In this experiment, the effects of the selective D2-like receptor antagonist haloperidol on this drug-free learning paradigm were tested.

Method

31 mildly food deprived pigeons were divided into three groups and treated with saline (group Sal, n = 12), 0.20 mg/kg haloperidol (group Hal-0.20, n = 12) or 0.25 mg/kg haloperidol (group Hal-0.25, n = 7). Haloperidol solutions were prepared by dissolving the drug in saline solution (0.9% NaCl). Pigeons of group Sal were injected with saline throughout, while

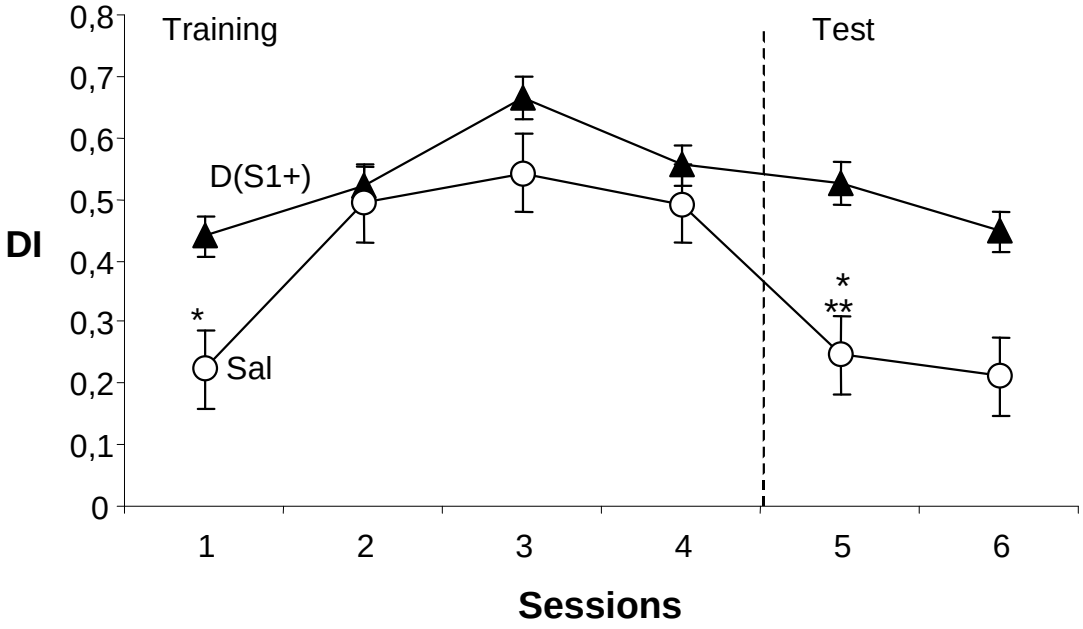


Figure 6.1 Average of DI values ± SE of groups saline. The Sal group contains pooled data from experiments 6 and 8. The D(S1+) group is the same group as in experiment 5. Statistical differences were significant in the first training session between both groups. An intra-group comparison was performed to evaluate the response decrement in the first test session. Statistical difference was only significant for the Sal group, between the 4th and 5th session (* signals inter-group and ** intra-group comparisons; $p \leq 0.05$).

pigeons in the other two groups were injected with the above mentioned haloperidol solutions during training, and with saline during test.

Results

Saline control group

The saline injected pigeons showed very low DI-values, both in training and test. A comparison of the Sal group with the D(S1+) group from the first phase of experiment 5 revealed a consistently lower performance of the Sal group (Figure 6.1). A two-factorial ANOVA showed a significant difference among sessions ($F_{5,120} = 5.17$; $p \leq 0.05$) and for the group factor ($F_{1,5} = 4.22$; $p \leq 0.05$). The further analysis of the decrement of the DI-values during test with Student t-tests for paired and non-paired samples between groups and between the 4th and 5th sessions (last training and first test session) for each group, revealed significant statistically differences. The analysis of the data showed statistically lower DI

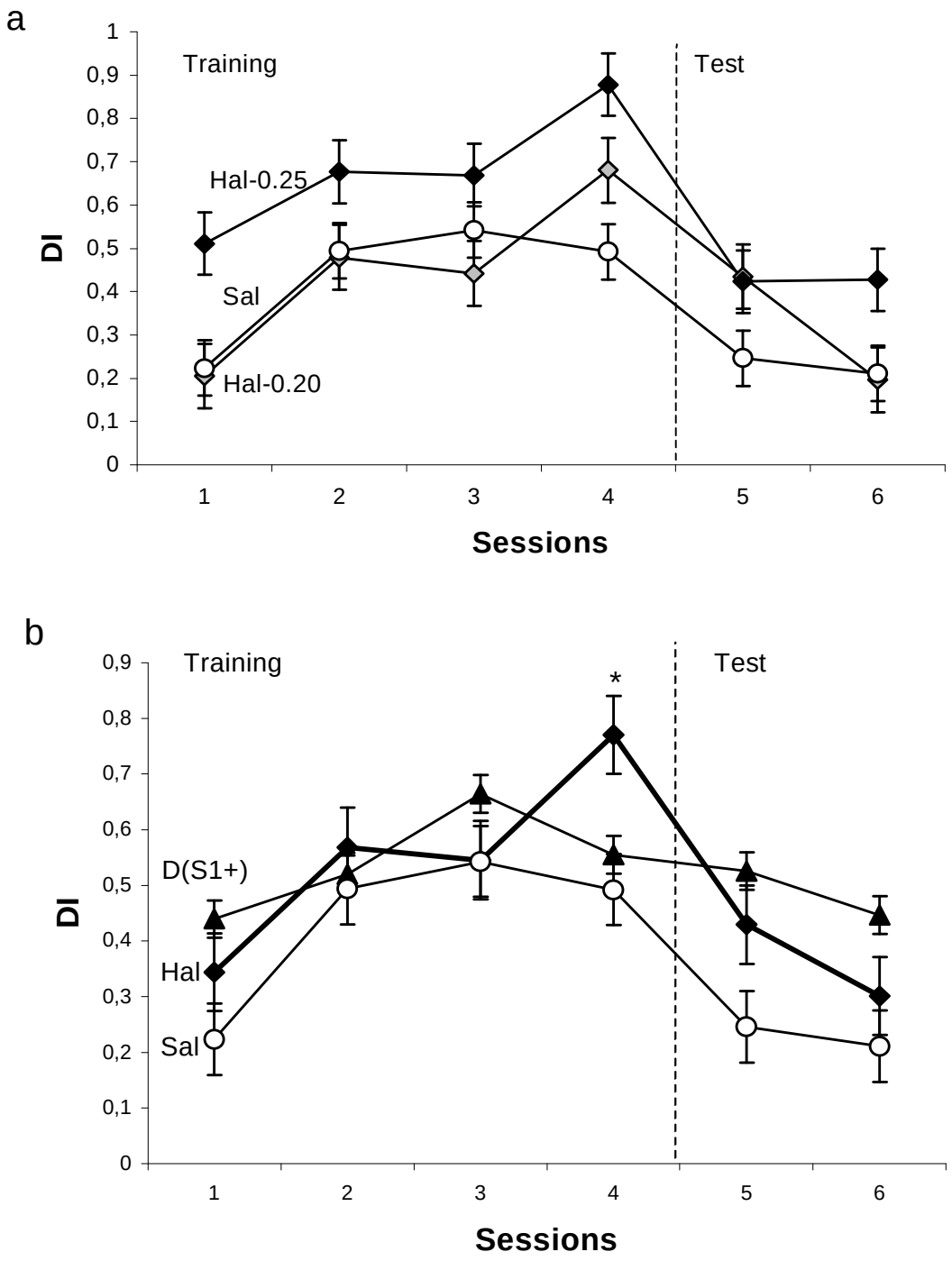


Figure 6.2. Average discrimination indices (DI) ± SE obtained in experiment 6. **a)** Groups Hal-0.20 and Hal-0.25 were injected with 0.20mg/kg and 0.25 mg/kg haloperidol respectively during training, and with saline solution during test. Group Sal was injected with saline throughout. No significant differences were found among groups. **b)** Hal-0.20 and Hal-0.25 groups were pooled and compared with Sal and D(S1+) (from experiment 5) groups. No statistical differences between Hal and D(S1+) were found. Hal was statistically higher than Sal group only in the 4th session. The decrement of the DI-values of the 5th session with respect to the 4th one, was statistically significant only for Hal and Sal groups (* signals $p \leq 0.05$).

value during the first training session for Sal than for the D(S1+) group (Student t-test for paired samples, $t_{29} = 2.15$; $p \leq 0.05$). There was no statistically significant difference for the D(S1+) group when the 4th and 5th sessions were compared (Student t-test for paired samples, $t_{10} = 0.25$; $p > 0.05$). The Sal group showed a significant decrement for the first test session (Student t-test for paired samples, $t_{14} = 2.99$; $p \leq 0.05$). This low performance of the Sal group was not expected at all. However, a theoretical explanation can be suggested on the basis of an arousal effect on task performance.

Briefly, the saline injection seems to impair the discrimination performance of animals. This is supported by the fact of the consistently lower performance of Sal compared with the D(S1+) group, as shown by Student test and by ANOVA-analysis ($p \leq 0.05$ for group factor). Additionally, the decrement of the DI values in Sal during test was not present in D(S1+) group. This latter result could be based on a possible frustrating non-reward effect. This will be explained later, in Discussion.

Haloperidol groups

Figure 6.2 shows the DI values obtained in experiment 6. Several pigeons had to be discarded because they did not fulfil the criterion of visiting the S+ pot at least once during training. Thus, three pigeons were discarded from the Sal group, six pigeons from the Hal-0.20 group and two pigeons the Hal-0.25 group. Additionally, the Sal group included pigeons of experiment 8, which had the same treatment (described above; n = 11). The final number of animals per group were: Sal, n = 20; Hal-0.20, n = 6; Hal-0.25, n = 5.

Haloperidol, just like other neuroleptics, is known to reduce spontaneous activity and to have a general sedating action (Barrett 1982, 1983; Korsgaard et al. 1985). In a preliminary experiment, it was found that haloperidol strongly reduces mobility in the pigeon depending on the dose (Godoy 2000). Similar results were obtained in the present work. For example: with injections of 0.3 mg/kg haloperidol, pigeons did not approach the pots (data not shown), and lower haloperidol doses (0.20 and 0.25 mg/kg) had some light impairing effect on the exploratory behaviour of some pigeons during training, which for this reason, were finally discarded (six and two pigeons discarded in Hal-0.20 and in Hal-0.25 groups, respectively). Despite this collateral effect, the remaining pigeons performed well during training of the discrimination task under the effect of haloperidol.

As it can be seen in figure 6.2a, DI-values of haloperidol injected pigeons did not reveal differences compared to the values of saline injected ones. The performance of those experimental subjects injected with the higher haloperidol dose (group Hal-0.25) was better

than that of subjects injected with the lower dose (group Hal-0.20). However, no statistical differences were found between both haloperidol groups (Hal-0.20 vs. Hal-0.25). To analyse the haloperidol effect on the discrimination task, both haloperidol groups were pooled and then compared with the saline treated group. No statistical differences between Hal and Sal groups were found, when a two-factorial ANOVA was performed (Figure 6.2b). Nevertheless, statistically significant differences among the sessions were found. A detailed analysis using Student t-test for paired samples showed that performance in the fifth session (first test session) was statistically lower than in the fourth session (last training session) of both Hal and Sal groups (Student t-test for paired samples, $t_{10} = 2.65$ and $t_{14} = 2.99$, for Hal and Sal groups respectively; $p \leq 0.05$). During test, haloperidol injected pigeons achieved higher DI-values than the saline control group, although the difference was only significant in the fourth session (Student t-test for non-paired samples, $t_{29} = 2.74$; $p \leq 0.05$). A similar comparison in the D(S1+) group of experiment 5, did not give statistically significant differences between groups, but among sessions, when a two-factorial ANOVA was performed, it did ($F_{5,100} = 4.02$; $p \leq 0.05$). This last group had similar treatment to the others excepting that no injection procedure was carried out. A detailed analysis of D(S1+) group using a Student t-test for paired samples did not reveal any statistical difference among sessions.

Discussion

The first attempt to provide an explanation of the arousal effect on the task performance was made by Yerkes and Dodson in 1908. As the Yerkes-Dodson law claims, “firstly, it was assumed that there was an inverted-U relationship between the level of tension, motivation or arousal on the one hand and performance on the other, with performance efficiency being optimal at some moderate level of arousal; secondly, strength of motivation or arousal was assumed to interact with task difficulty in such a way that the optimal level of arousal was inversely related to task difficulty” (Eysenck 1982). This law predicts interrelations among arousal, task difficulty and performance efficiency but it is uninformative with respect to the underlying mechanisms and processes.

Much later, other researchers suggested various notions about the possible mechanisms and processes responsible for such relationship among arousal, task difficulty and performance efficiency. In 1959, Easterbrook hypothesised that states of high emotionality, arousal and anxiety all produce comparable effects on cue utilisation. There is a progressive

reduction in the range of cues used as arousal increases, which in turn reduces the proportion of irrelevant cues employed, and so improves performance. When all the irrelevant cues have been excluded, further reduction in the number of cues employed can only affect relevant cues, and consequently the proficiency decreases (Eysenck 1982).

Broadbent, based on multiple experimental data, suggested in 1971 that a single arousal mechanism is probably insufficient. He postulated the existence of two interrelated arousal mechanisms. A "lower" mechanism involves the execution of well-established decision processes and it is affected by both arousal states, noise and sleeplessness. The sleeplessness is a low arousal state and elicits very cautious and unreactive decision criteria, whereas noise leads to a highly risk and hyper-active state. The "upper" mechanism monitors and alters the parameters of the lower mechanism in order to maintain a given standard of performance.

Some years later, Hasher and Zacks related the notion of variable attentional capacity to arousal. They put forward two closely related hypotheses concerning the effects of high arousal on task performance: first, high arousal impairs all processes making demands on attentional capacity, and second, high arousal has no effect on automatic processes (Eysenck 1982). Although there is not very much experimental evidence assessing these hypotheses, it is likely that they provide a relatively accurate explanation for experimental results in task performance

In summary, it seems that there is one arousal system which is affected primarily by intense or stressful external stimulation. This arousal system corresponds closely to the lower arousal mechanism postulated by Broadbent. If the arousal level is non-optimal, the second arousal system comes into operation. This second system compensates for the performance decrement produced by the first arousal system by re-allocating resources in response to task demands. This arousal system resembles Broadbent's upper arousal system (Eysenck 1982).

A possible mechanism for explaining the performance decrement under high arousal was proposed by Eysenck (1982). It consists mainly in a reduced ability to engage in parallel or shared processing. As a consequence, adverse effects of high arousal on performance are most pronounced in tasks requiring concurrent operations of various kinds (e.g. temporarily holding information, actively processing task stimuli and accessing long-term memory). Eysenck also argued that the reason for the reduced ability to process in parallel seems to be: a) there is a reduction in the total attentional capacity in high arousal, or b) total attentional capacity is not reduced, but smaller amounts of attentional capacity are available for task processing.

According to this theoretical background, the injection procedure seems to play the role of stressful external stimulus. This stimulus increases the arousal level in the pigeon by the time the animal is required to perform a discriminative task. So, in terms of the Yerkes-Dodson law, the cognitive performance is impaired by the high arousal level produced by the injection process. Animals that did not receive any injections are not stressed and the arousal level is supposed to be lower than in saline injected pigeons. The results showed in figure 6.1 are in agreement with this argument. Additionally, the decrement of the ID values during the first test session in the Sal group could also be explained by means of a non-rewarded frustration effect that could be added to the injection-induced stress. Consequently, we can expect that this non-rewarded frustration effect will lead to the lower decrement of DI values in the saline group. Weiner (1972) was the first who claimed that the cognitively perceived causes of failure (and of success) vitally affected performance. Nicholson and Gray (1972) assumed that the non-delivery of anticipated reward (i.e frustrative non-reward) was a punishing procedure, and additionally this punishment had an stronger negative effect on high anxiety people than on low ones. We assumed that the same effect could explain the difference observed between Sal and D(S1+) groups during test.

The results of experiment 6 where Sal and Hal groups showed a decrement of the DI values during test compared to non-interventive treated pigeons can be explained in the same way as for the Sal group. The Sal and Hal groups did not receive any food in the test phase, this seems to cause a frustrative non-reward effect. Following the same line, performance of the Hal group had to be impaired by the injection procedure. Nevertheless, the actual performance of the Hal group was better than the Sal one, because the tranquilliser effect of haloperidol. It is worth noting that the higher haloperidol dose produced a better performance than the lower one (figure 6.2a). As it was mentioned before, the blockade of the D2-like dopamine receptors is known to have this effect. Thus, the increased arousal by the injection was now depressed by the action of haloperidol on the D2-like dopamine receptors. Then, the performance of the Hal group during training was very similar to that of the D(S1+) group, which had only the disturbance of being handled by experimenters before to start the discrimination task.

Effects of SCH-23390 on the coloured grit discrimination

Experiment 7.

To test the effects of the selective D1-like receptor antagonist on this non-drug induced learning SCH-23390 had to be intracranially (i.c.) administered. Therefore, cannuli were bilaterally implanted in pigeons between the paliestriatum augmentatum and the paliestriatum primitivum (in the caudal striatum). In line with the associative learning model proposed by Wickens, the striatal area was chosen for the administration of this dopamine antagonist. This learning model basically involves the dopaminergic and glutamatergic interaction on striatal cells as the basis of associative learning. Additionally, administration of 1µl solutions only spread through a define area and not through the whole basal ganglia, where the striatum is located. Thus, cannuli implantation in the caudal area of the striatum was chosen because of the great extension of the striatum in the pigeon's brain. The location area of the cannuli are represented by the black dots on the schema of a pigeon's brain shown in figure 6.3 (adapted from Karten and Hodos 1967).

Method

The surgery and cannula implantation procedure, as well as the histological control, were described in the method section of experiment 4, chapter IV. As in that experiment, pigeons were allowed to recover from surgery for one week before experiment onset. SCH-23390 was dissolved in saline solution (0.9% NaCl) with 0.1% dimethyl sulfoxide (DMSO). The final volume of microinjections was 1µl per side, administered over a 2 minute period.

Twenty-four mildly food deprived pigeons, implanted with cannuli, were divided into four groups of 6 pigeons each. Group Sal received i.c. injection of vehicle (0.1% DMSO plus 0.9% NaCl in distilled water) during training and test. Group Sal/SCH3 received i.c. microinjection of vehicle during training and 3µg/µl SCH-23390 during test. Group SCH1 received i.c. microinjections of 1 µg/µl SCH-23390 during training and vehicle solution during test. Group SCH3 received 3 µg/µl SCH-23390 during training and vehicle solution during test.

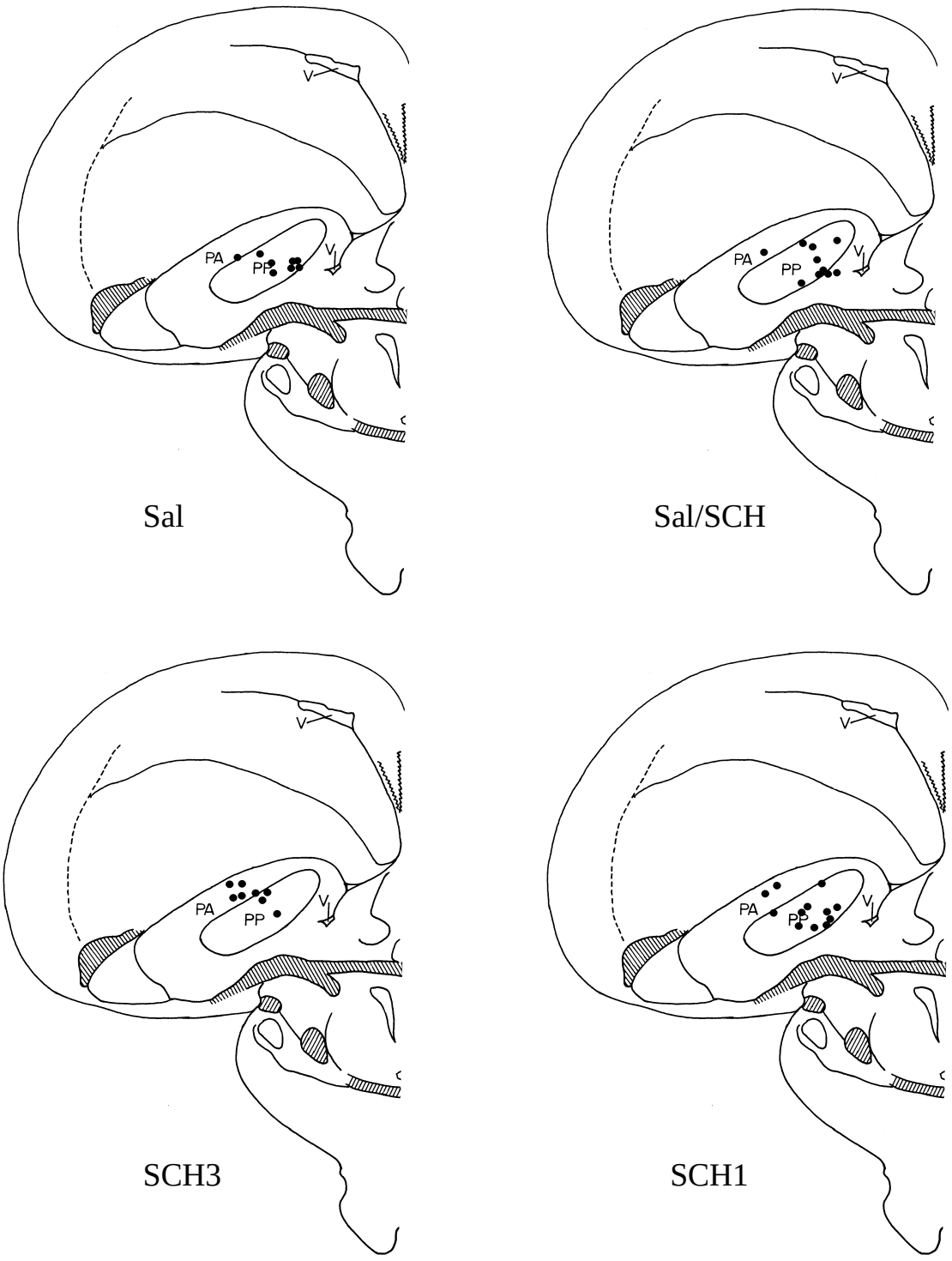


Figure 6.3 Schematic representation of the localisation of the tips in the posterior striatum at the antero-posterior level of 7.75. These correspond to pigeons included in the following groups: Sal (n=4), Sal/SCH (n=5), SCH3 (n=4) and SCH1 (n=6). For simplicity, all tips locations are shown on only one hemisphere side, (brain section diagram taken from Karten and Hodos 1967).

Results

The results of experiment 7 are shown in Figure 6.4a. Two pigeons of Sal and SCH3 groups and one from the Sal/SCH group were discarded, because histological control revealed that they had at least one of their cannula outside of the caudal striatum. Similar to the saline group of experiment 6 the i.c. injected saline pigeons in this experiment showed low DI values during training and test. The group D(S1+) was used as a reference for saline treatment (figure 6.4b). The Shapiro-Wilks normal distribution test showed that distribution of these values did not differ significantly from the normal one. So, to analyse the group Sal (n = 4) a parametric statistic was performed. A two-factorial ANOVA revealed statistical differences between D(S1+) and Sal groups ($F_{1,13} = 4.97$; $p \leq 0.05$). A detailed statistical analysis revealed that the DI values in the fifth session (first test session) significantly decreased compared with the fourth session (last training session, Student t-test for paired samples, $t_3 = 16.77$; $p \leq 0.05$). Additionally, DI values in the fifth session of the Sal group were statistically lower than those in the corresponding session for the D(S1+) group (Student t-test for non-paired samples, $t_{13} = 2.62$; $p \leq 0.05$). As in experiment 6, it can be concluded that the saline injection procedure impaired the performance of the discriminative task. The change in the arousal level possibly due to the injection procedure, could be responsible for this low performance, at least during the training phase. In test, another factor, the frustrative non-reward effect, could be added and as a result, a low training performance (not well learned discrimination task) led to low DI values.

Groups SCH3 and SCH1 did not differ statistically from each other when they were analysed with a one-factor ANOVA. The effects of the blockade of D1-like dopamine receptors in both groups were pooled and compared with Sal and D(S1+). The SCH (DI values of SCH3 and SCH1 together), Sal/SCH, Sal and D(S1+) groups are shown in figure 6.5. A two-factorial ANOVA of these groups' respones shows that values of the SCH group were significantly different from those of the Sal group. It also describes the difference among sessions (see figure 6.5a; $F_{1,12} = 11.02$, $F_{5,60} = 9.96$, for group and session factors respectively; $p \leq 0.05$). A detailed statistical analysis shows that DI values of the SCH group were statistically higher than those of the Sal group for the two last training sessions (Student t-test for non-paired samples, $t_{12} = 4.57$, $t_{12} = 3.34$, for the 5th and 6th sessions respectively; $p \leq 0.05$). Intra-group comparison showed that the decrement of the fifth session compared to the fourth was statistically significant in both SCH and Sal groups (Student t-test for paired samples, $t_9 = 5.81$, $t_3 = 16.75$ for SCH and Sal groups respectively; $p \leq 0.05$). The comparison

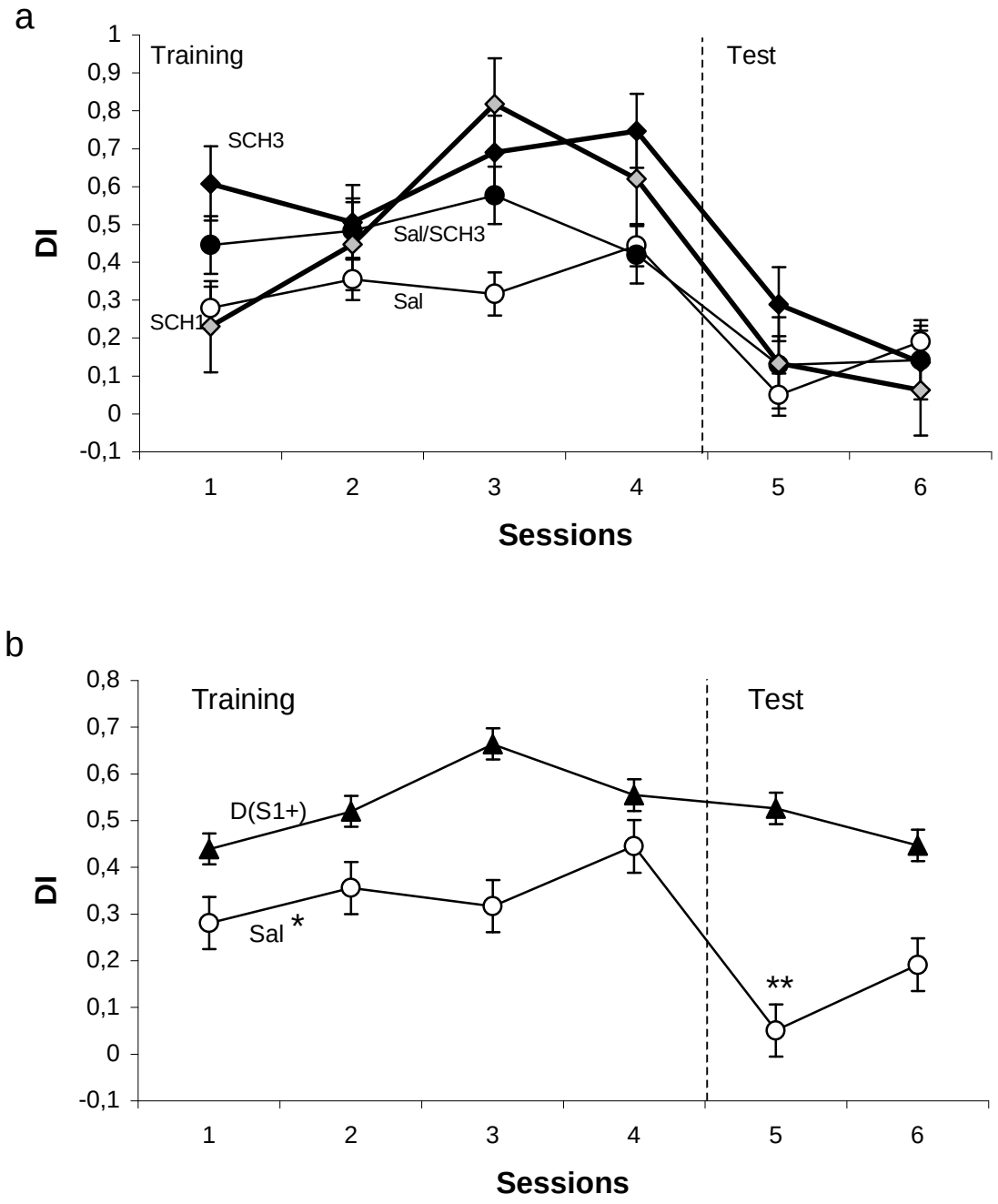


Figure 6.4 DI values ± SE of pigeons of experiment 7. **a)** Groups SCH1 (n=6) and SCH3 (n=4) received intracranial administrations of 1µg/µl or 3µg/µl SCH-23390 respectively during training and saline during test (fat lines). Group Sal/SCH (n=5) received saline during training and 3µg/µl SCH-23390 during test. Group Sal (n=4) received the saline solution throughout. No statistical differences were found between SCH1 and SCH3. For other comparisons see the main text and figure 6.5. **b)** Intra-cranial saline injected pigeons' performance compared with D(S1+) group performance of experiment 5. Statistical differences were found between groups. The decrement of the DI values of Sal was statistically significant between 4th and 5th sessions (* signals inter-group and ** intra-group comparisons; $p \leq 0.05$).

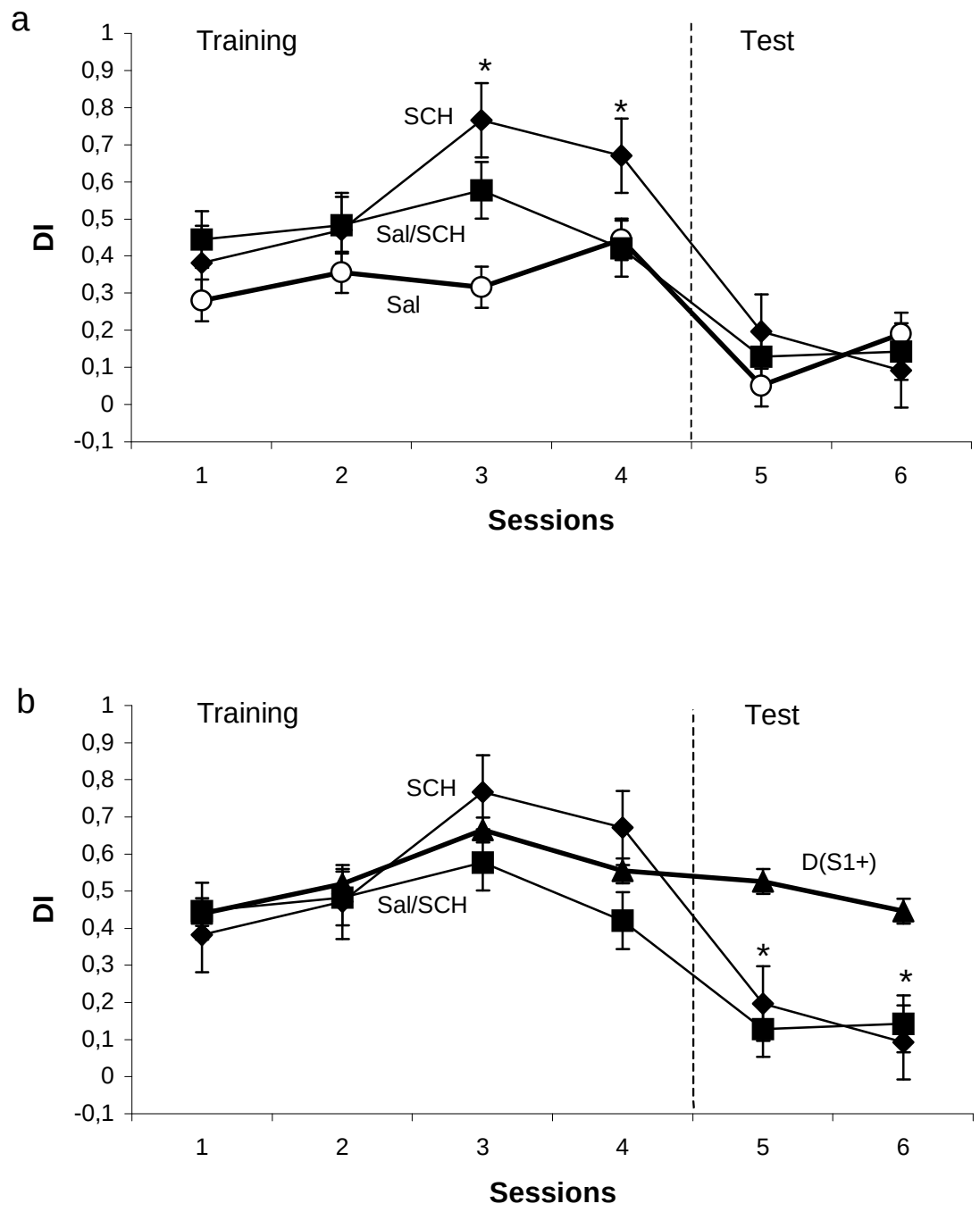


Figure 6.5. Results of experiment 7 are shown in DI values ± SE. a) Groups SCH (pooled data of SCH1 and SCH3), Sal and Sal/SCH are plotted together. SCH DI values were statically higher than Sal during the third and fourth sessions. No differences were found between Sal/SCH and Sal groups. b) Groups SCH, Sal/SCH are compared to D(S1+) group. Differences were found only during test sessions. Both groups, SCH and Sal/SCH had statistically lower DI values than D(S1+) during test (* signal $p \leq 0.05$).

between SCH and D(S1+) groups revealed no sigificant differences between groups, but significant differences among sessions when a two- factorial ANOVA was performed ($F_{5,95}$ = 8.89; $p \leq 0.05$). Nevertheless, a Student t-test for the test phase showed statistical differences between SCH and D(S1+) groups (Student t-test for non-paired samples, $t_{19} = 2.53$, $t_{19} = 2.97$, for 5th and 6th sessions respectively; $p \leq 0.05$). These findings suggest that SCH-23390 had an effect on the performance in the discriminative task similar to that of the D2-like receptor antagonist haloperidol. Both drugs seem to have a tranquillising effect on stressed pigeons. This effect improves the performance of animals during training. In the test, maybe an additional factor started to play a role. The decrement of responses in this phase can be explained by the frustrative non-reward effect which probably added to the injection-induced stress, decreasing the performance. Based on the results presented, it can not be argued that the blockade of D1-like receptors impairs the acquisition of colour discriminative learning. Despite of the fact that the local administration reduced considerably the action field of the drug, dopamine could be affecting the activity levels of some other brain area, i.e. the nucleus accumbens or the rostral striatum area.

The Sal/SCH group tested the effect of the D1-like receptor blockade during the retrieval of the discriminative learning. This group had an intermediate performance between Sal and D(S1+) groups during test. No differences between Sal/SCH and Sal, or between Sal/SCH and D(S1+) during training were found. However, the Sal/SCH group's performance deteriorated significantly during the first test session as compared to D(S1+) (Student t-test, for non-paired samples, $t_{14} = 2.38$; $p \leq 0.05$). This decrement of DI values in the fifth session was also statistically significant when compared with the fourth session (last training session; Student t-test for paired samples, $t_4 = 5.09$; $p \leq 0.05$). Despite the relatively high performance during training of the Sal/SCH group, the test phase was affected by the same factors as was the Sal group. The absence of differences between Sal/SCH and Sal, or between Sal/SCH and SCH groups, as well as the decrement of the test response of the Sal/SCH group with respect to D(S1+) group should be cautiously interpreted as an impairment of the retrieval process of the discriminative task. Further experiments are necessary to find out whether dopamine plays a role in the acquisition and retrieval of a colour discriminative task.

Effects of MK-801 on the coloured grit discrimination

Experiment 8.

In this experiment, the effects of the non-competitive, selective NMDA glutamatergic antagonist MK-801 were tested on this learning paradigm.

Method

24 mildly deprived pigeons were divided into four equally sized groups. Group Sal, was i.m. injected with saline during training and test. Group Sal/MK received saline during training and i.m. injections of 50µg/kg MK-801 during test. Group MK50 received i.m. injections of 50µg/kg MK-801 during training and saline during test. Group MK100 received i.m. injections of 100µg/kg MK-801 during training and saline during test. MK-801 was dissolved in saline solution (0.9% NaCl).

Results

Figure 6.6 presents the results of experiment 8. In order to increase the number of animals in the control group, the Sal group used here pools DI values from the Sal groups of experiment 6 and this one. As a consequence, the same interpretation and discussion of the Sal group results, as in experiment 6, can be done. Briefly, the Sal group showed lower DI values than the D(S1+), a first hand discrimination group, during the whole experiment. This decrement in the performance could be explained by the increment of the arousal levels induced by the injection procedure. As the Yerkes-Dodson law claims, the optimal performance of a cognitive task takes place at a moderate level of arousal. Higher or lower arousal levels result in a decrement in efficiency of performance. For that reason, it was of value the use a control group free of injection. The D(S1+) group from experiment 5 fulfilled this requirement and therefore was used to evaluate the effects of drugs on pigeons (Figure 6.6b). Despite of the low performance of the Sal group, it was also used in the evaluation of the drugs' effect. To asses the effect of the blockade of NMDA receptors on a discriminative task, both Sal and D(S1+) served as control groups.

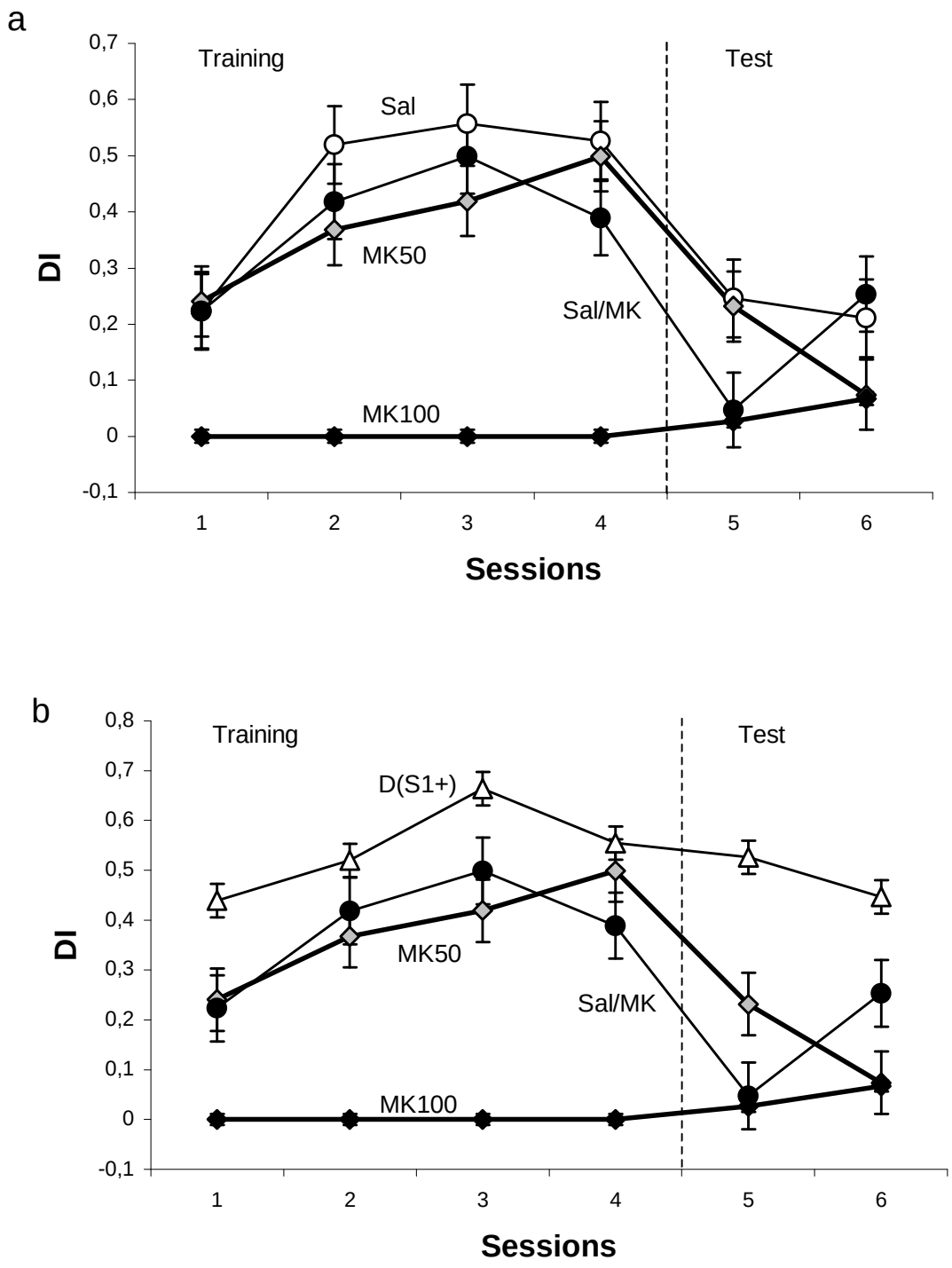


Figure 6.3. Averaged DI values ± SE of the pigeons in experiment 8. Group MK100 was trained with repeated i.m. injections of 100µg/kg MK-801 and tested with saline. This group evidenced a strong dyskinesia. Group MK50 was trained with repeated i.m. injections of 50µg/kg MK-801 and tested with saline. Group Sal/MK was trained with saline and tested with 50µg/kg MK-801 injections. The two later groups did not show significant differences when compared with group Sal, which was trained and tested with i.m. saline injections.

Pigeons in group MK100, trained with the largest MK-801 dose (100µg/kg), exhibited some dyskinesia (clumsiness) probably due to the high MK-801 dose. These animals, did not approach the pots and thus did not perform the discrimination task. It can be observed in figure 6.6a and 6.6b that the DI values of these pigeons stay near to zero during training, indicating that pigeons very rarely visited the S+. However, the DI values increased at a random level (about 0.02, data not shown) during test. In test, these animals were injected with saline instead of MK-801. This indicates that pigeons recovered their normal exploration activity. In fact, these pigeons did not have the opportunity to make any association between a given coloured grit and the presence of food. Thus, their performance during test was at a random level.

Group MK50 showed similar DI values to group Sal, especially in the last training and first test sessions, and no significant differences were found between these groups (figure 6.6a). There was also no statistical difference between MK50 and D(S1+) groups (figure 6.6b). However, the MK50 group showed a persistently lower performance than the D(S1+) group during training and test sessions. These findings suggest that MK-801 at this dose did not impair the performance in the discrimination task. The lower level of performance during training was not statistically different from the D(S1+) group. But, at the same time, it did not increase the performance, due to the increment of the arousal by the injection-induced stress. In contrast to the dopaminergic antagonist, no tranquilliser effects of MK-801 were observed during the experiment. The decrement of the DI values of the MK50 group during test (with respect to the last training session) was on the border of statistical significance, when the fourth and fifth sessions were compared (Student t-test for paired samples, $t_5 = 2.51$; $p = 0.054$), but it was statistically significant for the comparison of the fourth and sixth sessions (Student t-test for paired samples, $t_5 = 3.13$; $p \leq 0.05$). Similarly to the Sal group and during test, a new factor played a synergist role in the injection-induced stress. The frustrative non-reward effect depressed still more the DI values in the MK50 pigeons group.

Group Sal/MK also responded at the same level as the control group Sal (figure 6.6a). However, the decrement of the DI values during test was greater than for the Sal group. The decay in performance level in the fifth with respect to the fourth session was statistically significant (Student t-test for paired samples, $t_4 = 3.20$; $p \leq 0.05$). Despite that, there were not any statistical differences between Sal and Sal/MK groups during test, the DI values of the latter was close to the random level in the fifth session. The comparison of this group with the D(S1+) one reveals statistical differences in the fifth session. These results partially support the hypothesis that the NMDA receptors are involved in the retrieval of discrimination

learning. Additionally, the Sal/MK group showed that the saline injection had a synergist effect on the decrement of the DI values, together with the frustrative no-reward effect. The performance in the sixth session reached at the level of the Sal group. So the low performance in the fifth session was reversed.

These findings elicit some doubts about which factor was mainly responsible for the decrease in the DI values in the fifth session. There were at least three factors that could account for the decrement in performance in the fifth session. If the blockade of NMDA receptors was responsible, then the expected answer should be that performance was low because the effect of MK-801 on NMDA receptors impaired the retrieval during both test sessions. It can also be speculated that a novelty effect induced by the MK-801 administration could play a role. Additionally, the frustrative no-reward effect was not new and could also affect performance. In experiments 6 and 7 this frustrative no-reward effect was apparently homogeneous, since the performance of Hal and SCH groups always worsened diminished during test. The potential novelty effect induced by MK-801 or its action on retrieval mechanisms in discriminative learning were not properly tested in this experiment. Therefore, new experiments are needed that could adequately test these hypothesis. One possible experimental design could be that pigeons receive MK-801 injections in the morning and saline ones in the evening, performing the discrimination task only under saline effect during evening training. For test, the order of the injection should be inverted so that the MK-801 is now administrated in the evening, before performance of the discriminative task. The reverse group should be also carried out, MK-801 in the evening and saline in the morning, as well as pigeons performing the discriminative task under the MK-801 effect during training and saline during test. With this design, the MK-801 would not have a novelty effect when it is used in test phase.

General discussion

Since the dopaminergic and glutamatergic systems seem to be strongly involved in learning processes, it was expected that antagonists for one or the other system would impair the performance of pigeons in a learning task. The results of experiments six, seven and eight, where the effects of two dopaminergic selective D1-like and D2-like receptor antagonists and a glutamatergic selective NMDA receptor antagonist were tested, did not confirm this expectation. Some of the possible explanations for this negative result are that this colour discrimination task is not based on the dopamine and glutamate systems. To asses whether

these drugs do have an effect on this type of learning, a wider scanning of doses is necessary. Nevertheless, the doses used in experiments six and seven for haloperidol and SCH-23390 proved to have an effect on behaviour, increasing the performance during training of groups Hal and SCH respectively.

The blockade of both dopaminergic receptors, D1- and D2-like, showed similar results. Both groups showed an strong decrement of DI values in the fifth session compared to the fourth one. However, these decrements were due to artefacts of the technique and not to the drugs' effect itself. The arousal level was found to play a very important role during experimental performance. Stressful stimuli, like the injection procedure, produced in the pigeon such an arousal level that the efficiency of the performance in the cognitive task (colour discrimination learning) was impaired. Both dopaminergic antagonists had a tranquilliser effect. Therefore, the increment of the arousal level due to the stressful injection was reverted by the tranquilliser drugs' effect. The final results of experiments 6 and 7 were that both dopaminergic antagonists, instead of impairing the acquisition of learning, improved it. A second factor was added to the test phase. This factor, the frustrative no-reward effect, showed a synergist effect on the increment of arousal. Saline as well as with drug-treated pigeons showed the poorest DI values in the fifth session (first test session). For all cases, decrements were statistically significant (when the comparison was performed between fourth and fifth sessions).

Another possible interpretation of this result can be speculated, especially keeping in mind that further experiments using this paradigm should be set up. In pigeons, the potent dopaminergic agonist apomorphine, when injected in low doses, besides inducing stereotyped pecking, is known to induce anorexia (Deviche 1984). It could be speculated that dopaminergic antagonists (SCH-23390 and haloperidol) could exert the opposite effect. Thus, the mildly deprived pigeons used in this experiment would be more hungry when injected whit SCH-23390 or haloperidol than when injected with saline. This situation would facilitate exploration and activities related to feeding in pigeons injected with the dopaminergic antagonist, yielding the higher DI values observed in the experimental results of figure 6.2 and figure 6.5. No statistically significant difference was found with haloperidol or SCH-23390 when compared with the D(S1+) group during training. However, the response of pigeons treated with these drugs was frequently higher than that of those treated with saline (third and fourth sessions for SCH, and fourth session for Hal). Both alternatives, the tranquilliser and the hunger effect, are not mutually exclusive. Then, both alternatives could

give a reasonable explanation of these results. However, whether these speculations have a real basis, needs to be assessed in further experiments.

In experiment 8, the impairment effect on the discrimination task, e.g. blockade of the glutamatergic NMDA receptor associated channels was not seen with MK-801. The highest MK-801 dose used here induced collateral effects in that pigeons did not perform the task. Lower doses of this drug did not seem to have an effect on the pigeons' performance. In addition, the administration of the NMDA antagonist during the test phase did not give the expected results. The results of experiment seven were not clear enough to support or discard any of the proposed hypothesis that could explain the role of glutamate NMDA activity on colour discrimination. Further research is required with other NMDA receptor antagonists that do not have collateral effects. Possible candidates could be CGS-19755, a competitive NMDA antagonist, and also AP-5 and AP-7, which are very potent NMDA antagonists but do not cross the blood-brain barrier.

It is clear that more experimental results with these and other dopaminergic and glutamatergic drugs are required for the study the participation of these systems in learning. Some of these experiments are currently being done. Others will be the objective of future research. Emerging from the results presented in this chapter, an interesting issue to investigate deals with the effects of these dopaminergic and glutamatergic antagonists in the other learning paradigm used in this thesis, the apomorphine-context association. Experiments in that direction have been carried out and are presented in the next chapter. The comparison of the present results with parallel results obtained with this drug-induced form of learning may yield additional relevant information for future experiments.

Chapter VII

Role of dopamine and glutamate in apomorphine-induced learning

Introduction

The study of drug-induced learning has developed in parallel with the clinical study of several drugs. Morphine, heroine, alcohol, scopolamine, benzodiazepines, amphetamine among others, appear to have significant effects on behaviour. Studies have been specially focused on drugs tolerance. The term tolerance refers to the decrement in the efficacy of a given drug dose after repeated administrations. The development of drug tolerance during a chronic treatment makes it necessary to increase the dose in question in order to achieve its original effect. The most typical example of tolerance may be the consumption of ethanol in alcoholic drinks. The amount of ethanol that would make a casual drinker a bit tipsy is not likely to have any effect on a frequent drinker. Opposite to tolerance is the process of sensitisation. Sensitisation refers to the increase in the effectiveness of a given drug dose as a consequence of its repeated administrations. In recent years, among the drugs known to induce sensitisation, some psycho-stimulants, including apomorphine have attracted considerable attention. Two formal pharmacological rules for the above presented concepts are that while tolerance represents a shift of the dose-response curve to the right, a sensitisation represent a shift to the left.

Repeated injections of a constant apomorphine dose induce the gradual increment or sensitisation of the stereotyped pecking response of pigeons, until a maximum dose-dependent asymptotic level (Basten-Kreft 1977). This sensitisation seems to be based on a classical conditioning phenomenon (Godoy & Delius 1999, Lindenblatt & Delius 1987), as discussed in chapter III. Apomorphine also induces sensitisation of other behavioural responses in mice, rats and humans (Szechtman et al. 1987, Ljungberg & Ungerstedt 1977, Fekete et al. 1970). Briefly, when apomorphine effects (US) which elicit stereotyped pecking (UR) are repeatedly experienced within a certain experimental context (CS), a conditioned pecking (CS) develops in response to the context. The pecking sensitisation has been proposed (Godoy & Delius 1999) to emerge, because the conditioned pecking response (CR) would contribute to the unconditioned pecking under the effects of apomorphine, thus yielding an increased response. This apomorphine-context conditioning has been studied and

characterised in our laboratory, and has provided a useful tool for assessing the role of the dopaminergic system in learning.

As already mentioned, abundant experimental evidence indicates that the dopaminergic and the glutamatergic systems closely interact in the processes of learning and memory (Gruss et al. 1999, Greengard et al. 1991). It was therefore of interest to assess the effects of glutamatergic drugs on dopaminergic-dependent associative learning keeping in mind both apomorphine effects and context. Until now, no study with glutamatergic drugs had been done using this paradigm. Using the selective NMDA-receptor blocker MK-801, the experiments presented in this chapter assess the possible involvement of the glutamatergic system in this association. Additionally, the role of the dopaminergic system in this kind of learning was studied in more detail using the dopaminergic antagonists SCH-23390 and haloperidol, selective for the D1-like and for the D2-like receptors, respectively.

In our laboratory, Godoy (2000) carried out an experiment to test the effects of haloperidol (a selective D2-like receptor antagonist), on apomorphine-context associative learning. She addressed separately the acquisition of sensitised response and the expression of the already acquired sensitisation. Simultaneously injected with apomorphine, haloperidol was found to prevent the stereotyped pecking response induced by the dopaminergic agonist. Pigeons trained with both drugs simultaneously responded at the same level as saline injected controls. Additionally, since haloperidol counteracted the pecking effects of apomorphine (which constituted the US), no association with the context CS was expected to develop. Accordingly, pigeons trained with apomorphine plus haloperidol and those trained with saline responded at the same level when tested with apomorphine injections, developing a similar sensitisation curve. It was thus concluded that the co-administration of haloperidol and apomorphine during training impaired the acquisition of an apomorphine-context association. On the other hand, when pigeons had already been trained with repeated apomorphine injections in the experimental context, and developed a fully sensitised pecking response, the subsequent administration of haloperidol did not affect the expression of the conditioned response. Thus, after an apomorphine sensitisation training, pigeons tested with haloperidol responded at the same level as pigeons tested with saline.

On the basis of these results, experiments using the apomorphine-context associative paradigm were carried out to test the effects of the D-2 like receptor selective antagonist haloperidol (although with a different design compared to that of Godoy), the effects of the D1-like receptor selective antagonist SCH-23390, and the effects of the glutamatergic antagonist MK-801. The methodological details of the apomorphine-context conditioning, as

well as the experimental cage (EC cage, figure 3.4) used to train pigeons with this learning paradigm have been explained in chapter III.

The effects of haloperidol

Haloperidol, like other neuroleptics, is known to reduce spontaneous activity and to exert a general sedating action (Korsgaard et al. 1985, Barrett 1983, 1982). The above mentioned study by Godoy about the effects of haloperidol on apomorphine-context conditioning included a preliminary experiment designed to find a haloperidol dose which effectively counteracts the pecking effects of 0.5 mg/kg apomorphine, without reducing non-specific activity. She tested the effects of 0.2, 0.3 or 0.5 mg/kg injections of haloperidol alone on the amount of time pigeons spent inactive (as a measure of sedation), and the effects of the same doses on the total pecking response elicited by a co-administered 0.5 mg/kg apomorphine dose. The 0.5 mg/kg haloperidol dose significantly reduced the general activity of pigeons while the other two doses did not. On the other hand, 0.3 mg/kg haloperidol seemed to be more effective than 0.2 mg/kg in blocking the pecking effects of the co-administered apomorphine. In a subsequent experiment, 0.3 mg/kg were applied to assess the effects of haloperidol on learning.

However, there was still an important unanswered question about the possible unspecific effects of haloperidol that could effectively influence the results obtained with the apomorphine-context association: what are its effects on spontaneous pecking (not induced by apomorphine). Under normal conditions (i.e. without drug injection) it is hard to measure the effects of a treatment on spontaneous pecking because pigeons rarely peck. Thus, the measured response values would be so small that if any difference between haloperidol and saline treated pigeons exists, it would probably remain unobserved. A reliable way to induce the pigeons' pecking without drugs was to present food to food-deprived pigeons. In this way, a pecking baseline (not apomorphine-induced) could be obtained from hungry pigeons injected with saline. If a drug treatment induced any modification in the pecking response of deprived pigeons, the differences could be compared with a previously obtained baseline level. The next experiment described follows this approach, and in so doing tests the effects of haloperidol on pecking.

Effects of haloperidol on forage pecking

Experiment 9. This experiment was designed to test whether haloperidol affects the amount of spontaneous pecking of food-deprived pigeons rather them those treated with apomorphine. As mentioned, it was already known that relatively high haloperidol doses (such as 0.5 mg/kg) induce sedation. In this experiment, it was important to separate haloperidol effects on general activity from any possible effects on pecking. In other words, there was no need to study impaired pecking as a consequence of the haloperidol sedating effects. Instead, the main goal was to find any possible haloperidol effects on the neural circuits responsible for pecking. If such effects exist, they could be in principle either activating or supressing. A way to avoid haloperidol sedating effects is to use low doses. Godoy (2000) had found that 0.2 mg/kg haloperidol did not induce general sedation in pigeons. Thus, in this experiment, the effects of 0.15 and 0.25 mg/kg on pecking were assessed.

Method

Six pigeons were deprived (80% of their body weight) and maintained around that weight throughout the experiment. These pigeons were i.m. injected with saline for three daily sessions. Injections were administered in the home cage which had a pot containing 5 gr. of millet mixed with grit in approximately equal proportions. They were filmed for twenty minutes. Their response served as the baseline of pecking for food. Subsequently, the pigeons were divided into two groups and i.m. injected with haloperidol for four additional daily sessions. Group 0.25Hal and 0.15Hal received 0.25 mg/kg and 0.15 mg/kg haloperidol respectively. Videos were later analysed and the number of pecks per minute in the food containing-pot was recorded for five minutes after the first peck elicited by each pigeon.

Results and conclusions

Figure 7.1 shows the results of experiment 9. No significant differences were found when the responses of pigeons in groups 0.25Hal and 0.15Hal were compared with the baseline pecking values obtained during the first three sessions with saline injection. This outcome clearly indicates that neither a haloperidol dose of 0.25 mg/kg nor one of 0.15 mg/kg, affects the level of pecking exhibited by food-deprived pigeons.

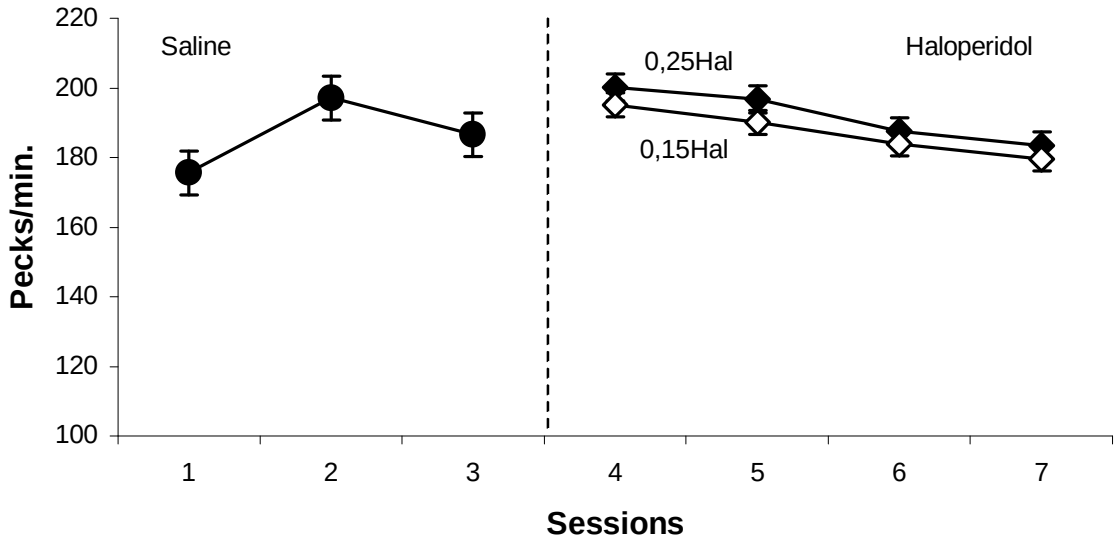


Figure 7.1. Average number of pecks per minute ± SE of pigeons in experiment 9 recorded during 5 minutes. In the initial three sessions, deprived pigeons had been injected with saline and allowed to feed. These values represent the baseline response. In the following sessions, pigeons were injected with either 0.25 or 0.15 mg/kg haloperidol and allowed to peck. No significant difference was found when the response of pigeons under the effects of haloperidol was compared with the baseline.

Effects of haloperidol on the apomorphine-context association

Experiment 10. This experiment was designed to assess the effects of haloperidol on the performance of pigeons trained with repeated apomorphine injections. Since in the former experiment 0.15 and 0.25 mg/kg haloperidol did not exert unspecific effects on the pecking response, an intermediate dose (0.20 mg/kg), was now used to assess its effects on learning. Additionally, in the earlier mentioned study, Godoy (2000) tested the effects of 0.30 mg/kg haloperidol on learning. Godoy commented in her conclusions that such a dose was a bit too small, because pigeons injected with that dose plus apomorphine displayed more pecking than saline injected animals, and that a larger dose would be more convenient. Thus, in this experiment, a second haloperidol dose of 0.35 mg/kg, was tested.

Method

The experiment consisted of a six training sessions and three test sessions. Eighteen naive pigeons were divided into three groups of six pigeons each. One group of pigeons received 0.35 mg/kg haloperidol during training and saline during test (group 0.35Hal). A second group received 0.35 mg/kg haloperidol simultaneously with 0.5 mg/kg apomorphine during training and saline during test (group HAL, capitals indicate large dose). A third group received 0.20 mg/kg haloperidol plus 0.5 mg/kg apomorphine during training and saline during test (group hal). Additionally, two control groups were used for comparisons. These control groups were the Ref-Apo and Ref-Sal groups presented in chapter III. As explained, to obtain standardised controls for apomorphine alone and saline alone treatments, the responses of a large number of pigeons trained and tested with apomorphine or saline were averaged over several months. Both control groups were also used for experiments 10 and 11 when necessary.

Results and conclusions

Figure 7.2 illustrates the results of experiment 10. Notice that in figure 7.2a the response values of all the groups, except group Ref-Apo, refer to the secondary Y-axis. It can be observed in this figure that the training responses of groups hal and HAL were lower than that of the control Ref-Apo, but higher than that of group Ref-Sal. The differences between the Ref-Apo and hal groups were significant for every session (Mann Whitney U test, $Z_{5,27} = 2.92$, $Z_{5,28} = 3.68$, $Z_{5,26} = 3.78$, $Z_{5,28} = 3.81$, $Z_{5,27} = 3.80$, $Z_{5,20} = 3.68$, $Z_{5,7} = 2.89$, $Z_{5,7} = 3.23$, $Z_{5,5} = 2.29$ for first to ninth sessions; $p \leq 0.05$). The differences between Ref-Apo and HAL groups were significant for every training session but not for test sessions (Mann Whitney U test, $Z_{5,27} = 2.69$, $Z_{5,28} = 3.55$, $Z_{5,26} = 3.69$, $Z_{5,28} = 3.81$, $Z_{5,27} = 3.80$, $Z_{5,20} = 3.69$ for first to sixth sessions; $p \leq 0.05$). Haloperidol was found to induce behavioural sensitisation to the dopamine agonist (Pudiak & Bozarth 1997, Pierce et al. 1995, Barnes et at. 1990). Thus, in general, the co-administration of haloperidol reduced the apomorphine pecking effects during training by blocking the apomorphine action on the dopamine receptors. Neverthelessm, it improved the pecking response during test, presumably by a hyper-sensitisation of the dopaminergic receptors. A further discussion about the possible mechanisms underlying the hyper-sensitisation phenomena will be offered in the next chapter.

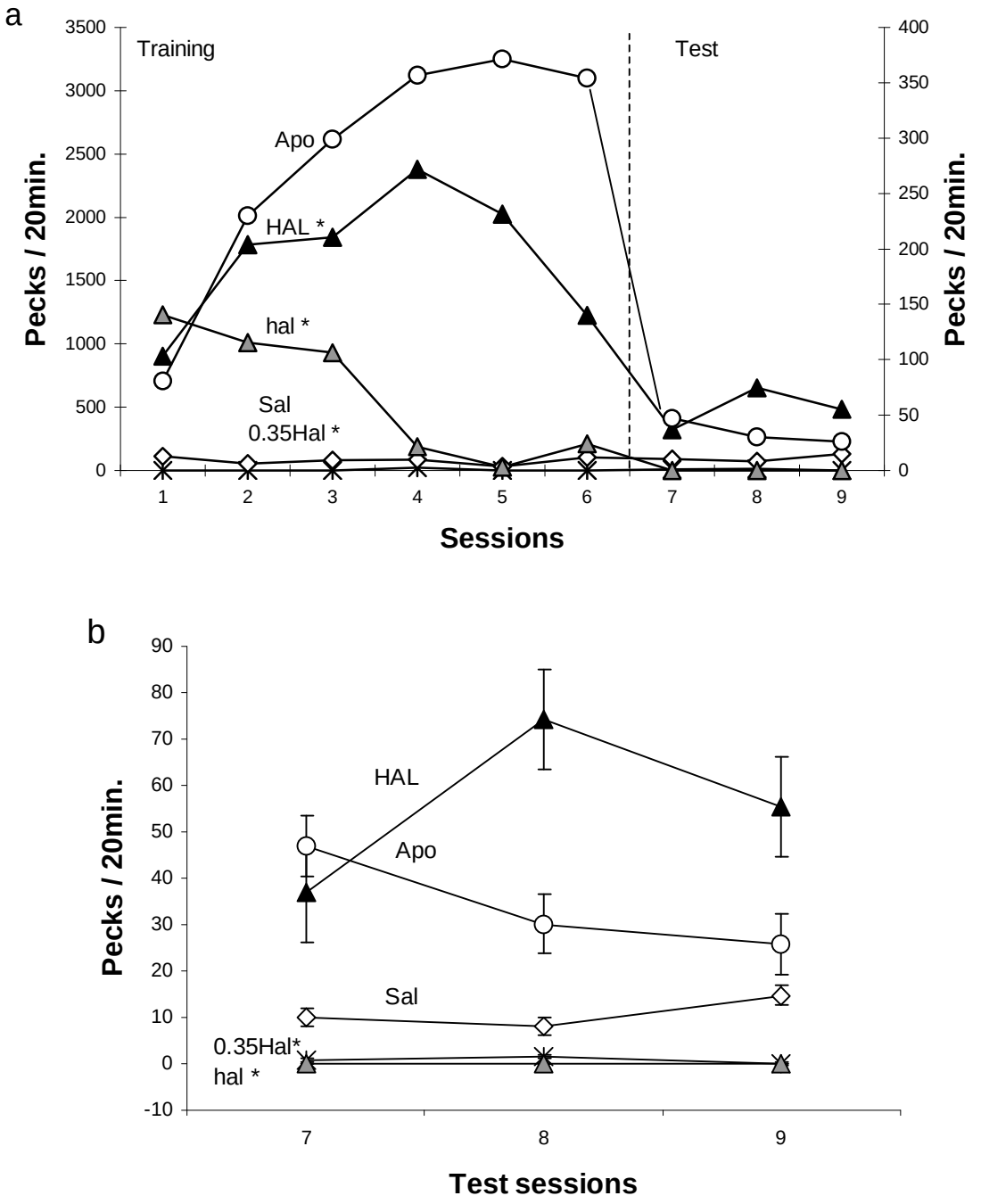


Figure 7.2. Averaged pecking response of pigeons in experiment 10. **a)** The pecking response values of group Ref-Apo, during training (open circles) refer to the primary Y-axis. The responses of group Ref-Apo during test, and of all the remaining groups during training and test refer to the secondary Y-axis. Statistically significant differences were found between groups HAL and Ref-Apo, between hal and Ref-Apo and 0.35Hal and Ref-Apo for every training session (* indicates $p \leq 0.05$). **b)** Averaged pecking response ± SE during the test (sal treatment) of experiment 10. Group hal responded at a significantly lower level than both Ref-Apo and Ref-Sal groups. The 0.35Hal group responded less than Ref-Apo in the seventh and eighth sessions (* indicates $p \leq 0.05$).

Surprisingly, the impairing effects of 0.20 mg/kg haloperidol on apomorphine-induced pecking were stronger than those of the 0.35 mg/kg dose. Since the apomorphine-effects constitute the US in this paradigm, it was expected that the haloperidol impairing effects would affect the development of an association between this US and the context CS. Group hal responded at a significantly lower level than the control Ref-Apo in every test session. This suggests that this 0.2mg/kg haloperidol had effectively blocked the acquisition of the US-CS association. It should be noticed however, that the pecking response of group hal fell significantly below the saline control level during test (Mann Whitney U test, $Z_{5,11} = 2.01$ for the seventh and eighth sessions and $Z_{5,9} = 1.98$ for the ninth session; $p \leq 0.05$). On the other hand, the test response of group HAL was higher than that of the control Ref-Apo, although the differences were small and not significant. This outcome suggests that the 0.35 mg/kg haloperidol could induce some hyper-sensitisation during training while the hal dose was too low to induce this phenomena. Thus, the apomorphine-induced pecking of HAL was higher than hal during training. This hyper-sensitisation lasted at least three days after the last haloperidol administration as can be concluded from the test phase results (figure 7.2b). Nevertheless, to asses this hyper-sensitisation hypothesis further experiments are necessary.

Group 0.35Hal responded at a lower level than group Ref-Sal in every session, although differences were only significant for the second training session (Mann Whitney U test, $Z_{5,11} = 2.01$; $p \leq 0.05$). This result suggests that haloperidol actually exerted some influence on general activity. Furthermore, haloperidol did not induce any pecking behaviour by itself.

The effects of SCH-23390

To test the effects of the selective D1-like receptor antagonist on apomorphine-context conditioning, SCH-23390 had to be intracranially (i.c.) administered. Therefore, cannuli were bilaterally implanted in pigeons between the paliestriatum augmentatum and the paliestriatum primitivum (in the caudal striatum). The locations of the cannuli are represented by the black dots on the schemes of a pigeon's brain, shown in figure 7.3 (adapted from Karten and Hodos 1967). The procedures for cannuli implantation, intracranial injections and histological control were described in detail in chapter IV.

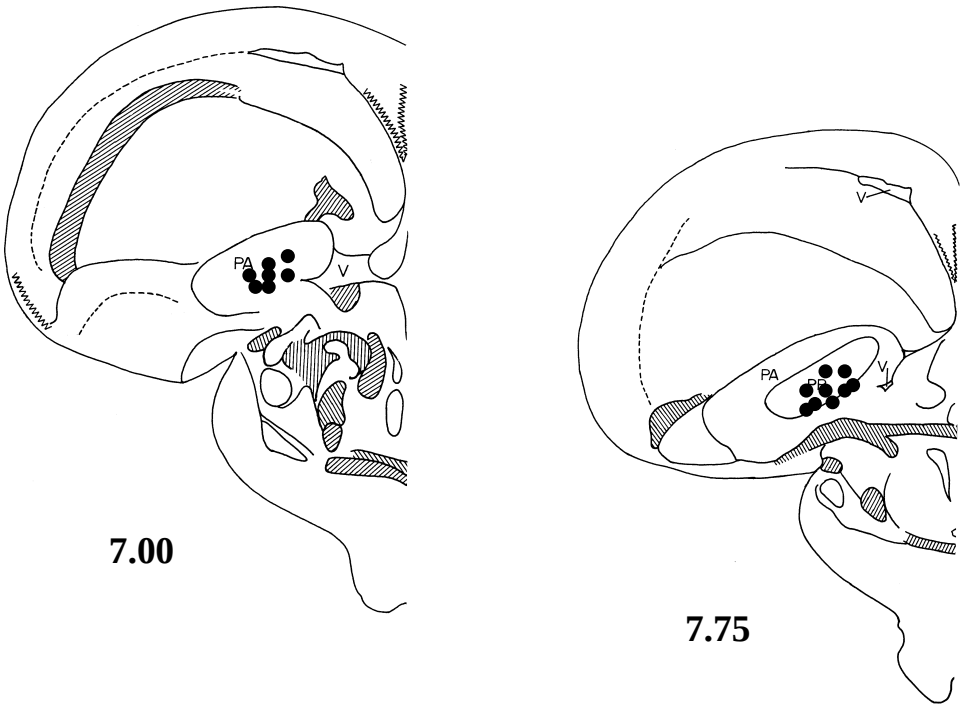


Figure 7.3. Location of the tips in the pigeon's brain, according to Karten and Hodos (1967). The cannuli were placed into the paleostriatum augmentatum (PA) and paleostritum primitivum (PP). Only one side of the brain is shown, at two different antero-posterior levels (7.00 and 7.75). Cannuli were actually implanted bilaterally at equivalent locations.

Determination of the effective SCH-23390 dose

Experiment 11. Before examining the effects of SCH-23390 on learning, it was necessary to determine a dose range effective in blocking the apomorphine-induced pecking. At the same time, it was important not to induce other unspecific behavioural effects or stereotypy, such as immobility or preening.

Method

Six cannuli-implanted pigeons participated in this experiment. They underwent two daily pre-test sessions of 30 minutes each, in which pigeons were administered SCH-23390 alone, to test the *per se* effects of this drug on their behaviour. Subsequently, the effects of the co-administration of i.c. SCH-23390 and i.m. apomorphine were tested for six daily sessions of 20 minutes each. As usual, after injections, the pigeons were placed in the EC cage and their behaviour was filmed.

For the two pre-test sessions, three pigeons received per hemisphere 1µg/µl SCH-23390 and three other pigeons 5µg/µl SCH-23390, injected directly into the striatal area. The final injected volume was 1µl per hemisphere. To analyse the effects of SCH-23390 on general behaviour during the two pre-test sessions, three variables were considered: 1) the spontaneous "number of pecks per session", 2) "activity", measured as the percentage of the time that pigeons spend exploring, preening or doing some other activity (i.e. all the time the pigeons were not passively sitting), and 3) "preening". A preening event was scored each time a pigeon touched the beak to its body and cleaned the feathers until it withdrew the beak from the body.

For the six test sessions, both groups received the corresponding SCH-23390 dose (1 or 5 µg/µl sch and SCH groups respectively; note that capital letters indicate a large dose). They also reveived the simultaneous i.m. injection of 0.5 mg/kg apomorphine. In order to save animals, no pigeons were implanted with cannuli for reception of i.c. saline solution in this experiment. Instead, three pigeons injected with i.m. saline from experiment 13 served as controls. The saline solution injected into the brain was at neutral pH and was assumed not to exert any effect by itself. The number of pecks per session elicited by these pigeons was analysed.

Results and conclusions

Figure 7.4 shows the results of the two pre-test sessions of experiment 11, when pigeons were administered either SCH-23390 or saline. The smallest SCH-23390 dose (1µg/µl) increased both levels of activity, preening and pecking. There were statistical differences when Sal and the smallest dose were compared (Mann Whitney U test, $Z_{2,2} = 1.99$, $Z_{2,2} = 1.96$, $Z_{2,2} = 1.96$ for pecking, activity, and preening variables respectively; $p \leq 0.05$). On the other hand, the behaviour of pigeons injected with the largest (5µg/µl) dose did not differ from saline injected control animals in any of the three variables, but differed from the smallest dose in pecking and activity variables (Mann Whitney U test, $Z_{2,2} = 1.96$ for both variables; $p \leq 0.05$). Thus, it seems that small doses of this D1-like receptor selective antagonist induce increased levels of general activity, while larger doses do not.

To analyse the blocking effects of SCH-23390 on the pecking induced by 0.5 mg/kg apomorphine, the number of pecks per session during six daily test sessions was considered. Figure 7.5 shows that the largest SCH-23390 dose (5µg/µl)strongly blocked the apomorphine-

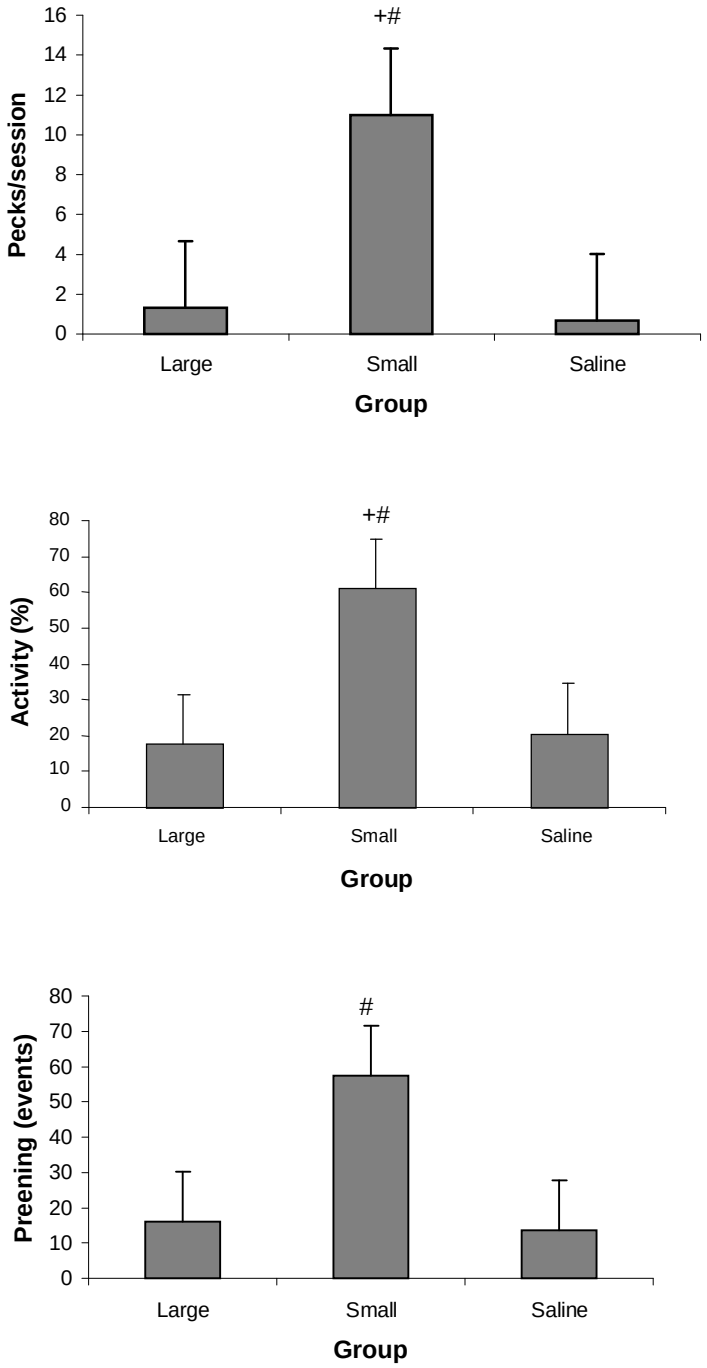


Figure 7.4. Averaged responses with SE of pigeons during the two pre-test sessions of experiment 11, when they were injected with a small (1µg/µl), a large (5µg/µl) SCH-23390 dose, or with saline. Three different behavioural variables were considered in assessing the effects of SCH-23390 *per se* on behaviour: number of spontaneous pecks per session, activity and preening (see main text). Pigeons receiving the smaller SCH-23390 dose showed an increment in all three behaviours. Those receiving the larger dose responded at the same level as did the saline controls. The symbol (+) indicates statistical differences ($p \leq 0.05$) between the effects of the largest and the smallest doses; significant differences between the effects of the smallest dose and saline are indicated by the symbol (#). No significant difference was found between the effects of the largest dose and saline.

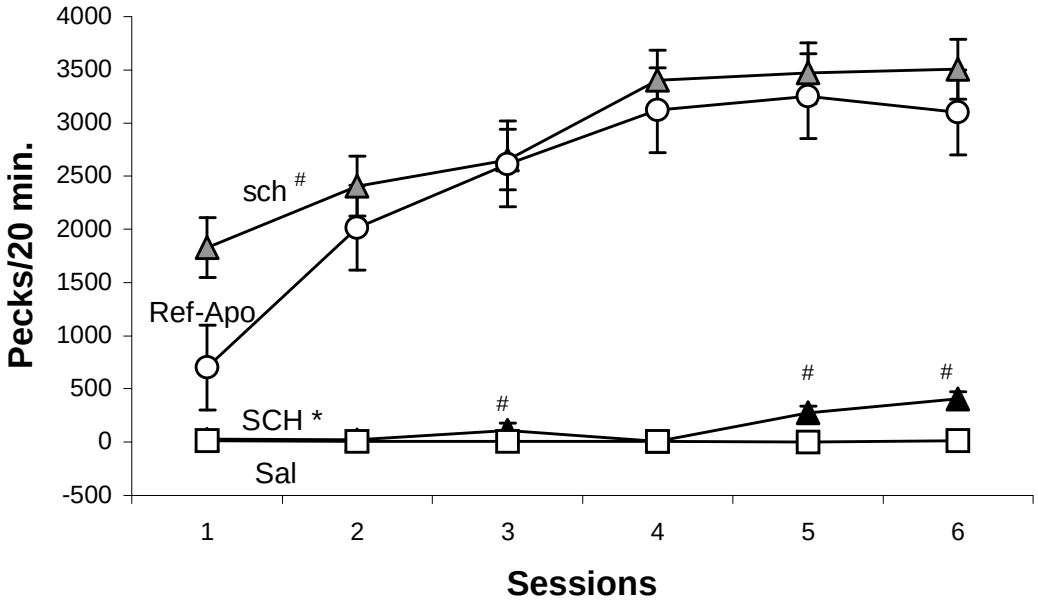


Figure 7.5. Results of experiment 11. Average ± SE of pecking response during the six test sessions. Additionally, the response of group Ref-Apo(TR) and Ref-Sal (see chapter III) is shown for comparison. Pigeons received bilateral i.c administrations of either a smaller (1µg/µl, sch) or a larger (5µg/µl, SCH) SCH-23390 dose (1µl per hemisphere) plus i.m. apomorphine, or saline solution. The symbol (#) indicates statistically significant differences ($p \leq 0.05$) between the SCH-23390 treated and Ref-Sal groups. The symbol (*) indicates significant differences ($p \leq 0.05$) between the SCH-23390 treated and the Ref-Apo groups.

induced pecking response, while the smallest dose did not. For comparison, the equivalent responses of the reference apomorphine- and saline-treated groups presented in chapter III, were also plotted in figure 7.5, as Ref-Apo and Ref-Sal, respectively. The response of pigeons treated with the smallest SCH-23390 dose (1µg/µl) plus apomorphine was higher than that of the control Ref-Apo(TR) group, though not significantly. On the other hand, SCH effectively blocked the pecking effects of apomorphine, since the pigeons treated with this dose plus apomorphine responded significantly lower than did the Ref-Apo, and at similar levels to the saline injected pigeons (Mann Whitney U test, $Z_{2,27} = 2.41$, $Z_{2,28} = 2.81$, $Z_{2,26} = 2.80$, $Z_{2,28} = 2.81$, $Z_{2,27} = 2.81$, $Z_{2,20} = 2.75$ for firth to sixth sessions respectively; $p \leq 0.05$). However, significant differences with the Ref-Sal group were found for the third, fifth and sixth sessions (Mann Whitney U test, $Z_{2,11} = 2.01$, $Z_{2,11} = 2.43$, $Z_{2,11} = 2.35$ for the third, fifth and sixth sessions respectively; $p \leq 0.05$). Thus, in the pre-test sessions without apomorphine, the SCH-23390 seemed to exert an activating effect on the animals' behaviour and during the test with

co-administered apomorphine, it did not block the typical pecking induction by this drug. On the other hand, the SCH group dose did not exert unspecific activation of behaviour, and it completely blocked the apomorphine pecking effects. However, in comparing the learning performance of saline versus drug-injected animals, it is normally better to use a drug dose which impairs the response to some extent than a dose which completely blocks it. With high doses that produce strong effects, more subtle, interesting differences can be overlooked. Therefore, an intermediate SCH-23390 dose, between 1 and 5 µg/µl, namely 3µg/µl, was used in the experiments that follow.

Effects of SCH-23390 on forage pecking

Experiment 12. As for haloperidol, it was necessary to examine the possibility that SCH-23390 could influence the outcomes obtained with the apomorphine-context association by exerting unspecific effects on the spontaneous pecking not induced by apomorphine. The same procedure as with experiment 9 was followed. Food-deprived pigeons were used in order to obtain a reliable, measurable pecking baseline (pecking for food) to compare with possible modifications in the pecking level under the effect of SCH-23390.

Method

Six pigeons were implanted with cannuli, as described in chapter IV, and deprived to 80% of their normal body weight. These pigeons underwent three daily sessions with i.c. administrations of saline and four subsequent daily sessions with i.c. administrations of 3µg/µl SCH-23390 (final injected volume 1µl per hemisphere). After receiving the drug, the pigeons were placed for 20 minutes in a home cage, with a pot containing 5 grams of millet mixed with grit in equal proportion. Their behaviour was filmed and the number of pecks per minute on the food containing-pot was recorded for 5 minutes after the first peck. After the experiment, the location of the cannuli were histologically corroborated as described in chapter IV.

Results and conclusions

Three pigeons were found to have at least one of the cannuli outside of the striatal structure (PA or PP according to Karten and Hodos 1967) and were grouped as the "Out" group. The

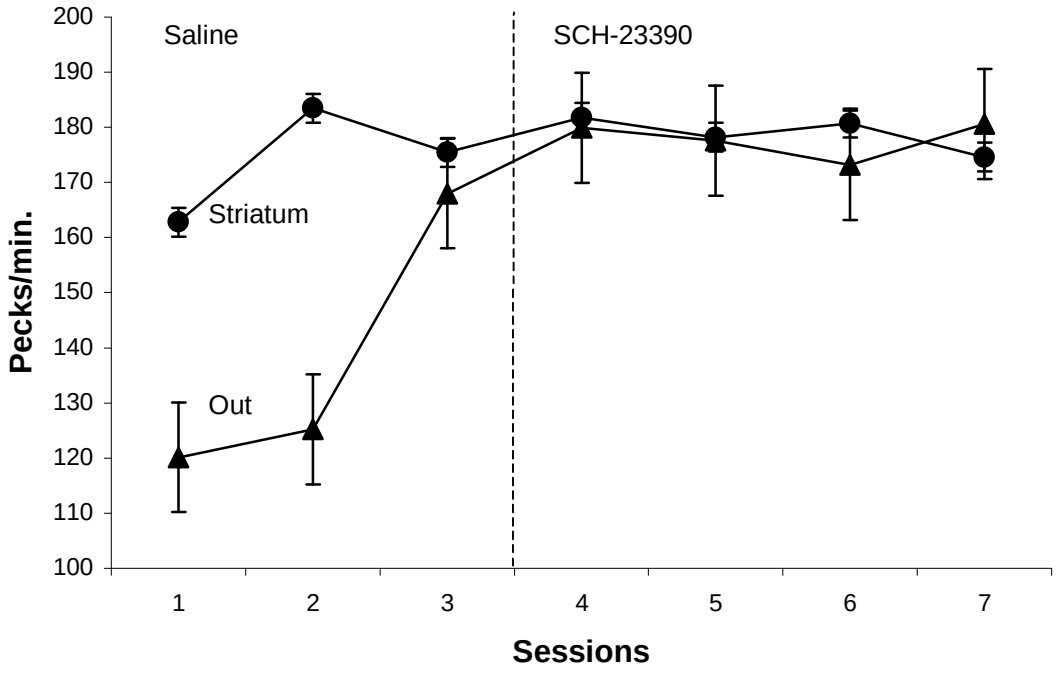


Figure 7.6. Averaged pecking response ± SE of the pigeons in experiment 12. Group Striatum (n = 3) had both cannuli implanted in the caudal striatum while group Out (n = 3) had at least one of the cannuli out side of this area. During the initial three sessions the pigeons received saline. Group Out pecked rather little during the first two sessions and recovered afterwards. In the final four sessions, both groups received 3µg/µl SCH-23390 (1µl per hemisphere). Their response under the effect of this drug did not differ from the last session of saline baseline.

remaining three pigeons had the cannuli correctly placed and were named "Striatum" group. The response of these groups during the first three sessions with saline served as a baseline for comparison with their performance during the subsequent sessions with SCH-23390.

The results of experiment 12 are shown in figure 7.6. No statistically significant differences were found between the baseline response level and the response under the effect of 3µg/µl SCH-23390 of Striatum group. This result suggests that such a dose of the dopaminergic antagonist did not affect the baseline level of pecking. Surprisingly, the response of the Out group in the initial two sessions with saline was rather low, although, in the last saline session and then when injected with SCH-23390, it reached a level similar to that of the Striatum group. One possible explanation for this outcome would be that the implantation of the cannuli in these pigeons unspecifically affected their behaviour. However, whatever the reasons for such minimal pecking under the effect of saline was, these pigeons recovered by the third session and increase it. A final conclusion of experiment 12 was that

SCH-23390 did not affect the pecking performance when administrated either to the caudal striatum or to some other area surrounding the striatum.

Effects of SCH-23390 on apomorphine-context association

Experiment 13. Based on the results of experiments 11 and 12, a dose of 3µg/µl SCH-23390 was used to assess the effects of this drug on apomorphine-context conditioning. This dose did not seem to affect pecking *per se* although it was expected to impair the pecking effects of 0.5 mg/kg apomorphine without completely blocking them. Even if the smallest dose tested (1µg/µl SCH-23390) did not block apomorphine-induced pecking, it was of interest to test its effects on apomorphine-context conditioning. This can be considered an attempt to investigate whether the effects of this drug on the response could be separated from its effects on learning. Cannula implantation, intracranial administrations, and histological controls were carried out according to the methodology described in chapter IV.

Method

Twenty- nine pigeons were implanted with cannuli for this experiment. Pigeons were i.c. administered bilaterally (1µl per hemisphere) with one of the SCH-23390 doses or saline, and i.m. injected with 0.5 mg/kg apomorphine or saline. Thus, each pigeon received both i.c. and i.m. injection of different solutions. Table 7.1 summarises the treatments received by each group. Group sch was trained with 1µg/µl SCH-23390 (i.c.) plus apomorphine (i.m.) and

Group name	**Training**		**Test**	
	i.c.	**i.m.**	**i.c.**	**i.m.**
Apo	Saline	0.5mg/kg apomorphine	Saline	Saline
Apo/SCH	Saline	0.5mg/kg apomorphine	3µg/µl SCH-23390	Saline
sch	1µg/µl SCH-23390	0.5mg/kg apomorphine	Saline	Saline
SCH	3µg/µl SCH-23390	0.5mg/kg apomorphine	Saline	Saline

Table 7.1. Drug treatments received by the pigeons in experiment 13. i.c. means intra-cranial administrations via cannuli implanted into the Striatum (1µl per hemisphere, see chapter IV), i.m. means intra-muscular injection (final volume 0.5 ml/kg, see method).

tested with saline (i.c. and i.m.); group SCH (capital words signal the largest SCH-23390 solution) was trained with 3µg/µl SCH-23390 (i.c.) plus apomorphine (i.m.) and tested with saline (i.c. and i.m.); group Apo/SCH received apomorphine (i.m.) and saline (i.c.) duringtraining and 3µg/µl SCH-23390 (i.c.) and saline (i.m.) during test and, finally, the control group Apo was trained with i.m. apomorphine and tested with saline. Six training and three daily test sessions were carried out. As usual after injections, pigeons were placed in the EC cage (described in chapter III, figure 3.4) where their behaviour was filmed for 20 minutes.

Results and conclusion

Analysis of the results showed that the co-administration of SCH-23390 and apomorphine produced a strong tendency to increase preening (a phenomenon not observed in experiment 11). Evidently, a too strong preening behaviour interferes with the amount of pecking that a pigeon elicits during a session. Since the aim of this study was to assess the effects of SCH-23390 on apomorphine-induced pecking behaviour, a criterion was fixed to discard those pigeons showing too strong preening. Videos corresponding to the first session were analysed and the preening spent time by each pigeon was recorded. Those subjects that spent more than 10% of the total time preening were not analysed. These birds however, underwent the whole experiment and their reaction to the drugs was recorded to be described elsewhere. Some other pigeons were also discarded *a posteriori,* because the histological analysis revealed that at least one of the cannula was incorrectly located, i.e. out of the caudal striatum. Results are presented for the final group sizes of: Apo (n=5), sch (n=4), SCH (n=2) and Apo/SCH (n=2). Unfortunately, not enough animals were left in some of the groups for statistical analysis of the data to be performed.

The results of this experiment are plotted in figure 7.7. The responses of groups Apo and Apo/SCH during training (when both underwent the same treatment of i.m. apomorphine and i.c. saline) are pooled (their response during test, when their treatments differed, are plotted independently). Notice that the response of all groups during test refers to the secondary Y-axis.

During training, responses of pigeons treated with the smallest SCH-23390 dose plus apomorphine were slightly lower than the responses of control pigeons treated with apomorphine and saline. Furthermore, responses of pigeons treated with the largest SCH-23390 dose were clearly lower than those of the apomorphine control pigeons. Thus, even

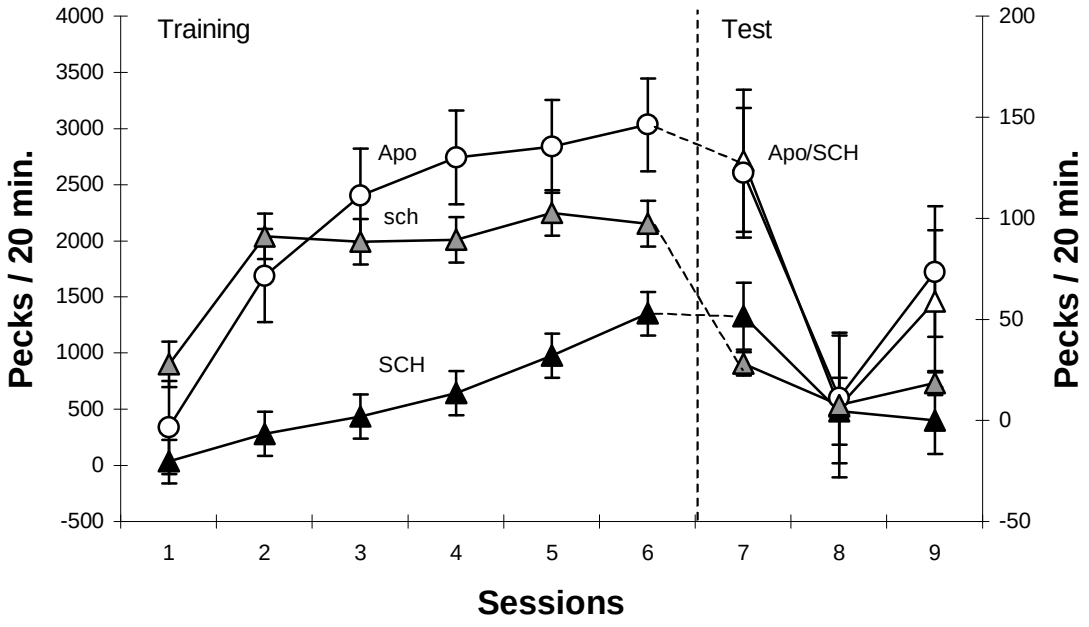


Figure 7.7. Averaged pecking response ± SE of pigeons in experiment 13. Groups sch and SCH were trained with 0.5 mg/kg i.m. apomorphine plus i.c. administrations of 1µg/µ or 3µg/µ SCH-23390 respectively and tested with saline. Groups Apo and Apo/SCH were trained with 0.5 mg/kg apomorphine (their training responses were pooled) and tested with saline or 3µg/µ SCH-23390 respectively (their test responses are plotted independently open triangles for Apo/SCH group). The sch group very slightly impaired the training response and did not impair the test response while the SCH group clearly impaired the apomorphine-induce pecking in training and the subsequent test response (compare with group Apo). The finding that group Apo/SCH responded at the same level than group Apo during test, indicated that, once the association had been established, 3µg/µl SCH-23390 did not affect its expression.

when the effect of 1µg/µl SCH-23390 was very slight, a dose dependent impairment by this drug on apomorphine-induced pecking was apparent. Responses of all groups during test were surprisingly low in the eighth session. Nevertheless, in the seventh and ninth sessions responses were as expected. There is no good explanation for the strong decrement in the eighth session. It could be an external factor that depressed the pecking response, since all the pigeons (independent of the treatment they underwent) were affected in the same manner and responded at a very low level, close to zero.

Since the apomorphine effects constituted the US in this paradigm, blocking them was expected to impair the association of these effects with the context EC cage. Accordingly, the conditioned pecking response of the SCH group during the subsequent test with saline, was

much lower than that of the control Apo group. This result indicates that the 3µg/µl SCH-23390 dose effectively impaired the apomorphine-induced pecking US, and consequently, the acquisition of an association between the pecking US and the context CS. The test response in the seventh session supports this argument. The sch response during training reached a lower asymptotic level than that of the Apo group, but these differences were not statistically significant. Nevertheless, the sch pecking response was statistically lower than the Apo response during the seventh session in test phase (Mann Whitney U test, $Z_{3,4} = 1.96$; $p \leq 0.05$). This indicates that 1µg/µl SCH-23390 was not enough to block the pecking response but in contrast, it was enough to block the acquisition of the apomorphine-context association. The later result, together with the weak pecking-impairing effects of this small dose during training and in experiment 11, when co-administered with apomorphine, suggests that 1µg/µl SCH-23390 was the dose limit for the blockade of the acquisition of the association between apomorphine effect and context without blocking the apomorphine induced pecking itself.

The test response of group Apo/SCH (trained with apomorphine and tested with 3µg/µl SCH-23390) achieved the same levels as the apomorphine treated controls. This result suggests that once the conditioned association between the apomorphine-effects US and the context CS has been acquired, the D1-like selective antagonist receptor SCH-23390 does not impair its expression. As a final conclusion, the blockade of the D1-like receptors prevents the acquisition of the association between the apomorphine effect and contextual cues. Conversely, it does not impair the retrieval of the conditioned response once, that association has occurred.

The next experiments were carried out to asses the role of the glutamatergic NMDA receptors on the apomorphine-context paradigm.

Effects of the MK-801

In the following experiments, the effects of the glutamatergic antagonist MK-801 on learning were tested. A first experiment was designed to assess the *per se* effects of different MK-801 doses on behaviour and at the same time to evaluate whether this drug induces stereotyped behaviour which can possibly interfere with the apomorphine-induced pecking response. As mentioned earlier, MK-801 readily goes through the blood-brain barrier, thus its effects can be assessed using intra-muscular injections.

Determination of the MK-801 dose

Experiment 14. This experiment was set up to evaluate the effects of several MK-801 doses on the general behaviour of pigeons. The objective was to describe the observed effects and to register the time elapsing between the injection and the maximum expression of the effects, as well as their total duration.

Method

Three pigeons were i.m. injected with three different increasing MK-801 doses, 0.1, 1.0 and 2.0 mg/kg. Each dose was injected once, with an inter-injection interval of two days. The observed MK-801 effects completely disappeared between 2 ½ and 3 hours after injection. These observations were made after the first MK-801 injection. On this basis, the inter-injection interval of two days was considered long enough to eliminate or extinguish all possible effects of MK-801 on the glutamatergic synapses. After injection, pigeons were returned to their home cages, where they were video taped for two hours (except for following the first injection. In this case the pigeons were observed for three hours. In all the subsequent sessions their behaviour was filmed for two hours). Video tapes were later analysed to describe the general behaviour of pigeons. Based on these observations, five parameters were considered relevant descriptors of the general effects of MK-801: head movements, wing movements, leg movements, stability when walking and effects on vision.

Results and conclusions

The two larger MK-801 doses (1.0 and 2.0 mg/kg) induced strong dyskinesia in pigeons. The animals evidenced impaired motor co-ordination of the head, wings and legs and a general instability when walking. The largest MK-801 dose (2.0 mg/kg), additionally produced impaired vision of the cage walls, and of objects. None of these effects was observed with the smallest 0.1 mg/kg dose. Table 7.2 summarises the observations of experiment 14. Based on these findings, MK-801 doses in the range of 0.05 mg/kg (50 µg/kg) to 0.12 mg/kg (120µg/kg) were chosen to study the effects of this drug on learning in subsequent experiments. The above described effects of the two higher MK-801 doses were clearly observed about 25 minutes after the injection. All these effects disappeared between 2 ½ and 3 hours later. This finding suggested that 30 minutes after injection is an adequate time

MK-801 dose (mg/kg)	Head movements	Wing movements	Leg movements	Stability when walking	Vision
0.1	not affected	not affected	not affected	not affected	not affected
1.0	impaired co-ordination	impaired co-ordination	impaired co-ordination	impaired	not affected
2.0	impaired co-ordination	impaired co-ordination	impaired co-ordination	impaired	impaired

Table 7.2. The effects of three MK-801 doses on the general motor behaviour of pigeons and on vision. Head, wings and legs movements as well as the stability at walking were strongly impaired by the two larger doses. 2.0 mg/kg also impaired vision. Only the smallest dose did not impair any of the behavioural descriptors.

interval for the evaluation of the effects of MK-801. Consequently, for the assessment of the effects of this drug on apomorphine-induced pecking, an interval of 30 minutes between the injection of MK-801 and the subsequent injection of apomorphine was chosen.

Effects of MK-801 on forage pecking

Experiment 15. In experiments 9 and 12, the possible effects of the D2-like and D1-like receptor selective antagonists haloperidol and SCH-23390 were tested in drug-free pecking. The same design used in those experiments was used here to assess the effects of MK-801 on pecking. With saline-treated and food-deprived pigeons would peck for food up to a given level. This was taken as a baseline. Thus, the observed modifications of that baseline as a consequence of the MK-801 injection can, in principle, be considered the result of drug effects on pecking performance.

Method

Six pigeons, deprived to 80 % of their normal body weight for the duration of the experiment, were injected with saline during three sessions and placed into a standard home cage with a single pot containing 5 grams of millet mixed with an equal amount of grit. Their behaviour in the cage was filmed. During the first 5 minutes after their initial peck into the pot, the number of pecking responses per minute for each pigeon was recorded. Their averaged response during these three initial daily sessions was considered the baseline. Subsequently,

the pigeons were divided into two groups of three pigeons each, and injected with either 80µg/kg or 120µg/kg MK-801. Thirty minutes after injection, they were put in the same cage and their responses were evaluated as explained before. The experiment run for four additional daily sessions.

Results and conclusions

The results of experiment 15 are illustrated in figure 7.8. The averaged response of the six pigeons during the initial three sessions, when they were injected with saline, was considered the baseline level of pecking for food-deprived pigeons. When responses of pigeons injected with either 80µg/kg or 120µg/kg MK-801 in the subsequent four sessions were compared with this baseline, none of the MK-801 doses seemed to impair pecking. The only observed difference was that the largest drug dose decreased the pecking level in the fourth session (first MK-801 injection). However, this was not statistically significant and response levels recovered for the next session. In general, the pecking response remained fairly constant at baseline levels throughout the experiment. Thus, MK-801 in the doses here tested can be considered not to exert *per se* any effects on the pecking response.

Effects of MK-801 on the apomorphine-context association

Experiment 16. In this experiment, effects of MK-801 on acquisition were assessed. The results of the two former experiments suggested a possible range of MK-801 doses, which would not have unspecific effects on pecking or on general behaviour. The two experiments also determined a suitable time interval after MK-801 injection for obtaining the maximum effects of this drug. In the following experiments, the influence of MK-801 on learning was assessed. Experiment 16 was designed to test the effects of this drug on the process of acquisition, when it was co-administered with apomorphine during a sensitisation training. Experiment 17 assessed drug effects on acquisition, when administered previous to the associative training, and the effects on the expression of the association, when administered after training.

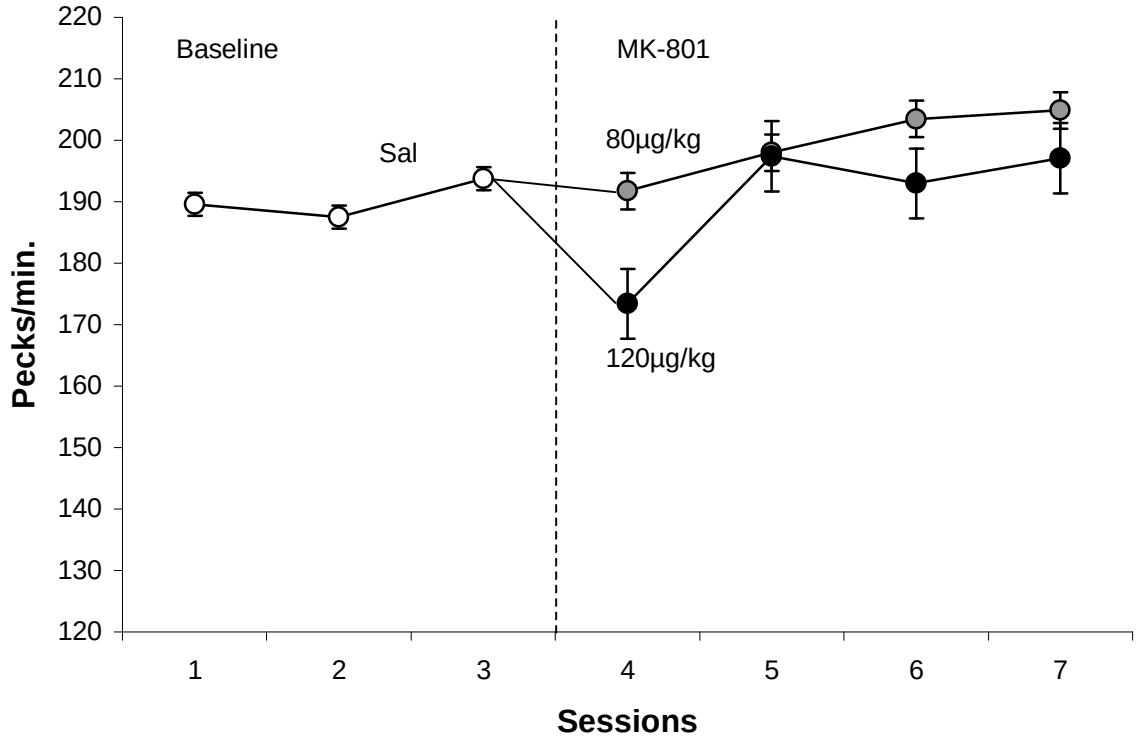


Figure 7.8. The average ± SE of pecking response of food-deprived pigeons in experiment 15. Six deprived pigeons were injected with saline and allowed to peck for food during three consecutive sessions. Their averaged pecking responses are plotted for the initial three sessions (left). This was considered a baseline. In the four subsequent sessions (right), three of these pigeons were injected 80µg/kg. The other three received 120µg/kg MK-801. Responses were then tested. No significant differences were found between MK-801 doses, nor between either dose and the baseline level.

Method

The experiment consisted of a six training and three daily test sessions. Fifteen pigeons were randomly assigned to three equally sized groups. For each training session, the groups were injected with 50, 80 or 120 µg/kg MK-801 (MK50, MK80 and MK120 groups respectively). Thirty minutes after MK-801 injection, pigeons received 0.5 mg/kg apomorphine. Immediately after apomorphine injection, pigeons were placed into the experimental EC cage (figure 3.4, chapter III) and filmed for twenty minutes as usual. For test sessions, all pigeons were injected with saline solution, placed into the EC cage and filmed as before. The responses of these groups were compared with those of the reference control groups treated with apomorphine (Ref-Apo) or with saline (Ref-Sal) presented in chapter III. Statistical analysis was carried out by means of a Mann Whitney U test.

Results and conclusions

Figure 7.9 shows the averaged pecking responses ± SE (only half bar plotted for visibility reasons) of experiment 16. As can be observed in this figure, the co-administration of MK-801 during training effectively reduced the pecking response induced by 0.5 mg/kg apomorphine in a clear dose-dependent manner. The training responses of pigeons injected with the smallest MK-801 dose tested, 50µg/kg, was significantly lower than that of group Ref-Apo in the second session ($Z_{4,28} = 2.02$; $p \leq 0.05$). Pigeons injected with the medium dose, 80µg/kg, responded even lower, and the differences with group Ref-Apo reached statistical significance in the first, second, fourth, fifth and seventh sessions ($Z_{4,27} = 2.96$, $Z_{4,28} = 2.60$, $Z_{4,28} = 2.07$, $Z_{4,27} = 1.96$ and $Z_{4,7} = 2.27$ for the respective sessions; $p \leq 0.05$). Pigeons trained with the largest dose (120µg/kg MK-801) responded significantly lower than group Ref-Apo in every training session ($Z_{4,27} = 3.52$, $Z_{4,28} = 3.43$, $Z_{4,26} = 3.35$, $Z_{4,28} = 3.53$, $Z_{4,27} = 3.51$ and $Z_{4,20} = 3.42$ for sessions one to six respectively; $p \leq 0.05$). These dose-dependent differences were mirrored when each MK-801 treated group was compared with the saline injected control group. The higher the MK-801 dose, the smaller the differences from the Ref-Sal group. Statistical significance are not indicated in figure 7.9 in order to avoid graphic confusion. However, pigeons injected with 50µg/kg MK-801 responded significantly higher than those of the Ref-Sal group in every training and in the first test session ($Z_{4,11} = 2.65$, $Z_{4,11} = 2.29$, $Z_{4,11} = 3.34$, $Z_{4,11} = 3.18$, $Z_{4,11} = 3.34$, $Z_{4,11} = 3.34$ and $Z_{4,11} = 2.43$ for the first to seventh sessions; $p \leq 0.05$), those trained with 80µg/kg responded significantly higher from the fourth to the sixth and in the eighth session ($Z_{4,11} = 2.14$, $Z_{4,11} = 2.51$, $Z_{4,11} = 2.51$ and $Z_{4,11} = 1.99$ for respective sessions; $p \leq 0.05$). Finally, those trained with 120µg/kg MK-801 responded significantly higher, but only in the sixth training and eighth test sessions ($Z_{4,11} = 2.28$ and $Z_{4,11} = 2.48$ for the sixth and eighth sessions respectively; $p \leq 0.05$).

The previous results of experiment 15, which demonstrated that MK-801 did not have effects *per se* on pecking, together with the observation in this experiment, that MK-801 impaired the apomorphine-induced pecking, demonstrated that this glutamatergic antagonist can readily block at least some of the apomorphine-effects. Since the apomorphine effects constituted the US in the apomorphine-context association, the acquisition of such an association was expected to be impaired by MK-801. Consequently, responses of MK-801 trained groups were expected to be lower than those of the Ref-Apo group when tested later with saline injections. This expectation was confirmed in the first test session (session seven). In this session, the Ref-Apo group showed the highest response while all the MK-801 trained

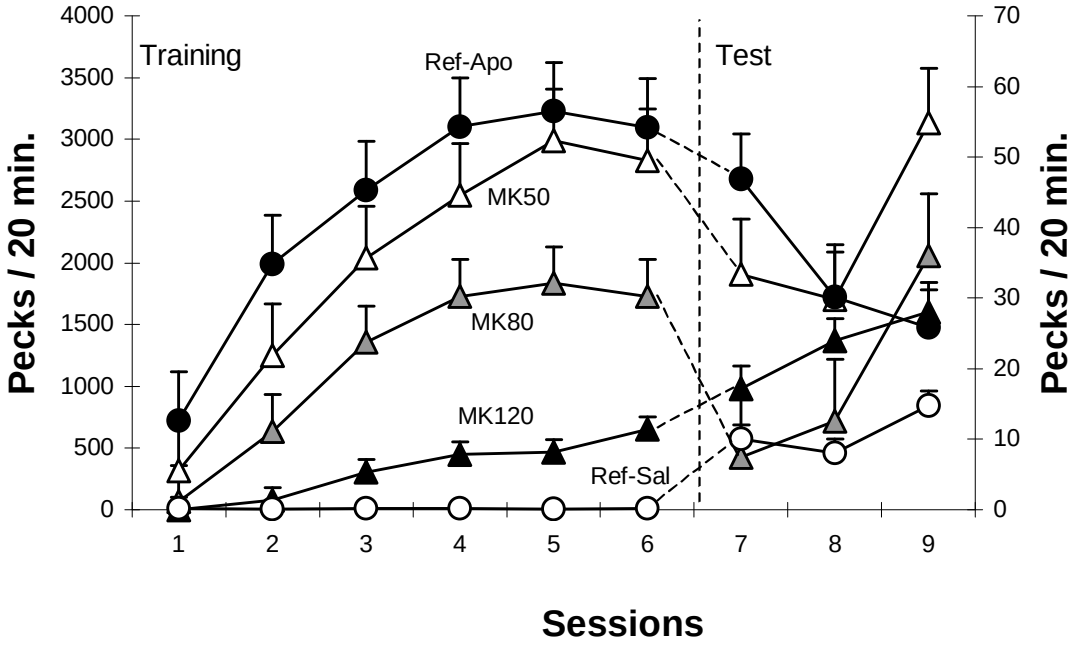


Figure 7.9. Averaged pecking responses ± SE (half bar) of pigeons in experiment 16. Pigeons were trained with different MK-801 doses plus apomorphine and tested with saline. Those trained with 50µg/kg MK-801 (MK50) responded significantly lower than the control Ref-Apo in the 2nd and 4th training sessions. Those trained with 80µg/kg MK-801 (MK80) responded significantly lower in the 1st, 2nd, 4th, 5th and 7th sessions. Pigeons trained with 120µg/kg MK-801 (MK120) responded significantly lower in every training session. MK50 group responded significantly higher than group Ref-Sal in every training session, while MK80 responded significantly higher from the 4th to the 6th and in the 8th session. The MK120 group responded significantly higher than the Ref-Sal group, but only in the 6th training session. The symbol (*) indicates $p \leq 0.05$ for the comparisons with the Ref-Apo. In order to avoid confusion, significant differences with the Ref-Sal group are not indicated.

groups showed lower responses. The first test response with saline, after an apomorphine sensitisation training, best indicates the expression of a possibly acquired apomorphine-context association in these pigeons. The conditioned response is known to diminish very quickly in subsequent test sessions. This phenomenon was observed for the Ref-Apo group. Such a fast extinction of the learned conditioned response, as well as the fact that the magnitude of the first test responses are normally much smaller than the last training ones, is thought to be the outcome of a complex US use. That is, the apomorphine effects, include

diverse effects on pecking and also on perception and/or internal states (Godoy & Delius 1999). Thus, the first test responses are normally assumed to be the most reliable indicator of the magnitude of the conditioned pecking.

It is however worthy of mention that the strongest impairment on the acquisition of the conditioned pecking seemed to be exerted by 80µg/kg MK-801. The group under this treatment showed the only significant difference with Ref-Apo in the first test session. Surprisingly, the response of all the MK-801 trained groups, independent of the dose being used, increased in the course of the repeated test sessions with saline. Actually, responses of the three groups in the third test sessions were higher than that of the control group Ref-Apo. Differences between the first and the third test sessions were not significant for any of the MK groups with respect to the Ref-Apo group (excepting the already mentioned first test session of MK80 group). The smallest MK-801 dose induced the strongest response increment during test, although no differences between MK-801 trained groups and Ref-Apo ones reached statistical significance. The test responses of the MK groups were higher than those of the saline injected control group Ref-Sal. Significant differences were found for pigeons injected with 50µg/kg MK-801 in the first test session ($Z_{4,11} = 2.43$; $p \leq 0.05$), for pigeons trained with 80µg/kg MK-801 in the second test session ($Z_{4,11} = 1.99$; $p \leq 0.05$) and for pigeons trained with 120µg/kg MK-801 also in the second test session ($Z_{4,11} = 2.48$; $p \leq 0.05$). A detailed discussion about pecking response increments of MK groups during test will be offered in the general discussion.

The blockade of the glutamatergic NMDA receptors by means of MK-801 dose-dependently impaired the apomorphine-induced pecking. Mechanisms underlying the blockade of the apomorphine-induced pecking do not seem to exert a direct impairment effect on the performance of pecking itself, as demonstrated in experiment 15. This conclusion is based on the hypothesis that MK-801 could block apomorphine-induced pecking. A speculative argument could be that MK-801 could act on apomorphine-induced pecking by affecting the dopamine action at the reward or motivational level that in turn is a component of the association between the apomorphine effect and contextual cues. The acquisition of the apomorphine-context association was also impaired. However, the effects on acquisition were not so clearly dose-dependent as compared with the effects on the pecking response (as can be observed in the test phase). In the general discussion of this work (next chapter) detailed arguments will be offered that could explain the outcome of the MK groups in this phase.

Effects of MK-801 on the expression of the apomorphine-context association

Experiment 17. In this experiment, the effects of MK-801 on the process of acquisition were tested by administering the drug previous to training. Its possible effects on the expression of an already acquired association were also tested by administering the drug after training.

Method

The selection of a MK-801 dose that would have an remarkable effect on the apomorphine-context association was based on the previous results of experiment 16. In this experiment, a dose of 80 µg/kg MK-801 had an intermediate inhibition effect on pecking responses, while a 120 µg/kg dose had a very strong blocking effect on the same behavioural responses. For the experiment to follow a 100 µg/kg dose would be chosen. Twelve naive pigeons were randomly allocated into two equally sized groups. Group Apo-MK underwent six training sessions with 0.5 mg/kg apomorphine and three test sessions with MK-801. Group MK-Apo underwent six pre-training sessions with MK-801, six training sessions with apomorphine, and three test sessions with saline. As usual, the experiment was carried out in consecutive daily sessions. Immediately after apomorphine or saline injections, and 30 minutes after those of MK-801, pigeons were placed in the EC cage (figure 3.4, chapter III) and filmed for 20 minutes. For comparisons during this experiment, test responses of the reference control groups, Ref-Apo and Ref-Sal presented in chapter III, were used.

Results and conclusions

Figure 7.10 shows the results of this experiment. In order to simplify the graph and because during pre-training with 100 µg/kg of MK-801 the responses of pigeons remained at a constant level near zero, only the last three pre-training sessions were plotted. Thus, the first session in the left side of the figure corresponds to the fourth pre-training injection. During training, MK-Apo and Apo-MK groups responded at a very similar level, and no differences between them were found. Since group Apo-MK received apomorphine without any previous treatment, it displayed a typical sensitisation curve and the observed acquisition of an apomorphine-context association was assumed to be normal. This despite the fact that the Ref-Apo group reached an asymptotic level higher than both Apo/MK and MK/Apo groups. It

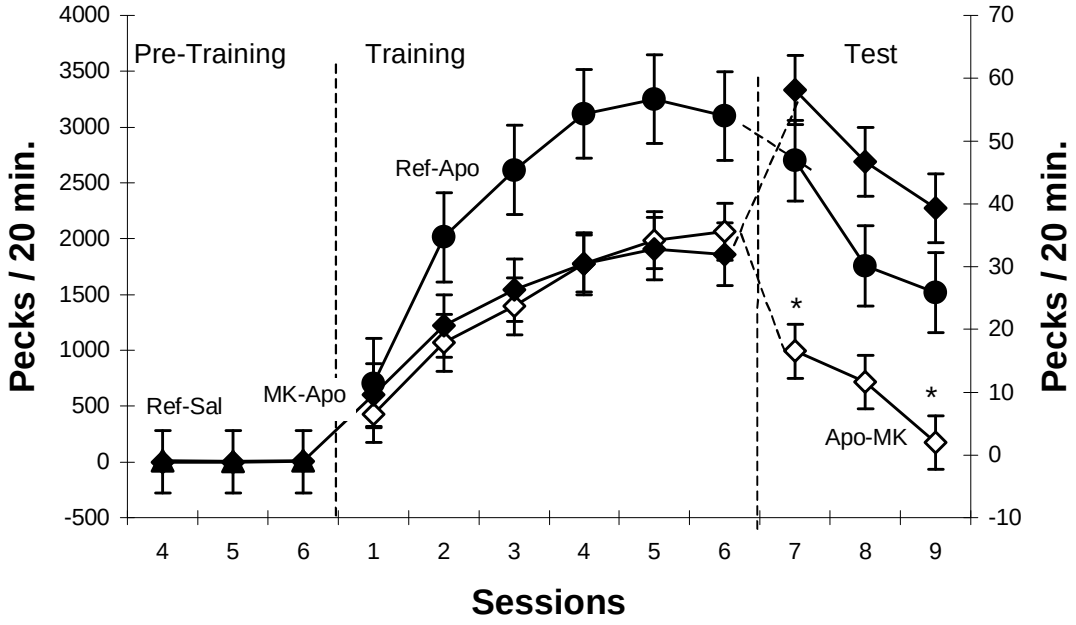


Figure 7.10. Averaged pecks per session ± SE of groups in experiment 17. Training responses of the Ref-Sal and training and test responses of the Ref-Apo groups were plotted. Group MK-Apo was pre-treated with six injections of 100µg/kg MK-801 (only the last three pre-training sessions are shown, starting in session 1), trained with 0.5 mg/kg apomorphine and tested with saline. Group Apo-MK was not pre-treated. The training consisted of injection of the same apomorphine dose and test with a 100 µg/kg MK-801 dose. Pre-training and training refer to the primary Y-axis on the left; the test refers to the secondary Y-axis on the right. No differences between the pre-training response of MK-Apo and the responses of Ref-Sal groups were found, suggesting that MK-801 does not induce pecking response by itself. No differences between MK-Apo and Apo-MK groups during training were found suggesting that previous MK-801 treatment does not impair the development of sensitisation. The Apo-MK groups however, responded lower than the Ref-Apo in the first and third test sessions (+ indicate $p \leq 0.05$), suggesting that MK-801 impairs the expression of an already acquired apomorphine-context association.

was of value to make the comparison between Apo/MK and MK/Apo rather than to compare the Ref-Apo and MK/Apo groups. The fact that the training responses of group MK-Apo, pre-trained with MK-801, did not differ from those of Apo-MK group, suggests that a previous treatment with this NMDA antagonist did not influence the subsequent apomorphine-induced pecking, and consequently it does not seem to affect the acquisition of the apomorphine-context association.

In the test phase, the response of group MK-Apo, now injected with saline, was higher than the response of the control Ref-Apo group, also under saline treatment. However, the differences were not significant. Despite the fact that pecking responses of the MK-Apo group during test decreased as the number of sessions increased, the response level corresponded to the increased response of the MK groups of experiment 16. A more detailed explanation of this fact will be offered in the general discussion in the next chapter. Group Apo-MK, now injected with 100µg/kg MK-801, responded lower than group Ref-Apo. Differences reached significance in the first and third test sessions (Mann Whitney U test, $Z_{6,6} = 1.95$ and $Z_{6,5} = 196$ for seventh and ninth sessions respectively; $p \leq 0.05$). This later outcome suggests that 100µg/kg of the glutamatergic antagonist MK-801 was effective in blocking the expression of an already acquired association between the effects of the dopaminergic agonist apomorphine and the contextual cues.

The next chapter will contain a general discussion of all the experimental results of this work. Anatomical, biochemical and behavioural experiments will be summarised and discussed from the point of view of learning. Some arguments that could explain the experimental results in this chapter will be offered in a more detailed way.

Chapter VIII

General discussion

General discussion

The main objective of this thesis is to asses the roles of the glutamatergic and dopaminergic systems in associative learning. In order to fulfil this endeavour, two associative learning paradigms were used. One of them involved a drug-induced learning while the other one employed a simultaneous visual discrimination paradigm. The experimental results of chapter VII unequivocally support the hypothesis that both glutamate and dopamine neurotransmitter systems play a role in the learning process of the apomorphine-context association. Since the dopaminergic and glutamatergic systems are involved, and probably interact with each other, during the learning process (see general introduction), the influence of different antagonists on the acquisition (both dopaminergic and glutamatergic drugs) and the expression (only glutamatergic drugs) of the apomorphine-context conditioning used here show a striking concordance with results obtained in previous works (Godoy & Delius 1999, Cox & Westbrook 1994, Martin-Irvenson & Reimer 1994, Beninger 1993, Druhan et al. 1993, Hamamura et al. 1991, Hoehn-Saric et al. 1991, Welsch-Kunze & Kuschinsky 1990).

The results of these experiments support certain conclusions. As demonstrated in experiment 2, the apomorphine-context association can be long lasting (as long as two years). This paradigm also produces modifications in the proportion of D1/D2 like receptors, as demonstrated by means of the binding assay technique in experiment 3. However, the brain area in which dopamine acts is still not clear. Experiment 4 intended to clarify this point. Here, it was found that apomorphine failed to exert any stereotyped pecking behaviour when locally administered into the striatum. But, when injected into the nucleus accumbens, it was effective in producing stereotyped pecking behaviour. However, has still not been demonstrated that the pigeon's nucleus accumbens is responsible for dopamine activity associated with the learning processes. Gargiulo and colleagues failed to modify discriminative learning in pigeons by administering apomorphine into the nucleus accumbens (Gargiulo et al. 1998). These findings suggest that even if the nucleus accumbens is involved in learning, other nearby brain regions are probably involved as well. Therefore, the finding of experiment 13, that dopaminergic D1-like receptor antagonists affect apomorphine-context conditioning when locally administered in the dorsal striatal area (which lie dorso-anterior to

the nucleus accumbens), was not completely surprising. Both dopaminergic antagonists, the selective D1-like receptor SCH-23390 and the selective D2-like receptor haloperidol, exerted similar effects on this associative learning, as demonstrated in experiments 10 and 13. These drugs proved to block the acquisition of a new association, but not the expression of an already acquired one. On the other hand, the glutamatergic antagonist MK-801, selective for NMDA receptors, impaired both the acquisition of new association and the expression of an already acquired one (experiments 16 and 17). Even when these results seem reasonably clear, it is worthwhile to discuss the implications of this outcome as well as inferences about possible interactions between both systems. The unspecific effects of the drugs here tested also deserve some discussion.

In the second learning paradigm used, a simultaneous colour discrimination procedure was developed in which animals had to associate a given colour with a food reward (experiment 5). Although this discrimination task was a good paradigm for the examination of behavioural responses, it failed as a tool for the assessment of drug effects on learning. Clearly, this discrimination task was too sensitive to external factors such as situation induced stress (i.e. the injection procedure) or the tranquilliser effect of receptor antagonist drugs as demonstrated in experiments 6, 7 and 8. These results lead us to be more careful in designing further experiments involving this type of discrimination task.

The anatomical experiments performed on the pigeon spinal cord (experiment 1) revealed a well-defined dopaminergic system. Nevertheless, the functions of the dopaminergic cells in the spinal cord of pigeons still remain unclear. A promising hypothesis for further clarification could be that in the dopaminergic system, at the level of the spinal cord, the cells contacting the central canal liquor work mainly as a sensor or detecting system that accumulates dopamine and/or other metabolites from the cerebrospinal fluid (Smeets et al. 1991, Smeets & Gonzales 1990). Other cell populations could transmit sensory signals to the brain through the DARPP-32 neurones in the animal's dorsal horn. The same DARPP-32 neurones could also play an inter-neurone role in the control of sympathetic reflexes (Horn & Kohli 1992, Lewis & Coote 1990). The question whether DARPP-32 neurones receive input either from dopaminergic neurones in the spinal cord and/or from the diencephalo-spinal system could not be answered. Despite the lack of evidence regarding dopamine existence in these ganglion cells, it is possible that the DARPP-32-immunoreactive cells from the dorsal horn receive projections from the dorsal root ganglion. The localisation and number of the DARPP-32-immunoreactive cells in the dorsal horn could indicate that they receive their sensory input from the dorsal root ganglion. But again, this remains speculative.

Unspecific drug effects

Experiments 9, 11 and 12 were designed to estimate of doses of the dopaminergic antagonists haloperidol and SCH-23390 that do not exert unspecific effects on the general or the pecking behaviour of pigeons. Experiments 14 and 15 were designed for an equivalent evaluation of the glutamatergic antagonist MK-801.

Dopaminergic antagonists have long been known to produce motor quiescence (Beninger 1983). Among them, the selective D2-like receptor antagonist haloperidol (depending on the dose), has frequently been found to reduce subject performances in different species, in several appetitive learning paradigms, (Korsgaard et al. 1985, Barrett 1983, 1982). An earlier study (Godoy 2000) demonstrated that a dose of 0.5mg/kg had unspecific sedating effects on pigeons, motor activity and spontaneous pecking. Conversely, a lower haloperidol dose (range 0.2 - 0.3 mg/kg) did not.

The unspecific effects of SCH-23390 had never previously been evaluated in pigeons. Thus, in experiment 11, two SCH-23390 doses, 1µg/µl or 5µg/µl (1µl per hemisphere), were tested for their effects on the general behaviour and motility of pigeons. General behaviour and motility were measured by means of three parameters: the number of pecks per session, the percentage of time pigeons spent inactively (sitting), and the number of preening events. It was found that the smallest SCH-23390 dose (1µg/µl) augmented all three behaviours, while the largest dose (5µg/µl) did not induce effects any more or less than the control saline, in any of the three chosen variables. Thus, it seems to be that an inverse dose-response relationship between unspecific motor activity and doses of SCH-23390 exist. Subsequently, as a first analysis of the SCH-23390 blocking effects on apomorphine-induced pecking, the same SCH-23390 doses were co-administered with 0.5 mg/kg apomorphine. The largest SCH-23390 dose strongly blocked the apomorphine-induced pecking response, while the smallest dose did not. Thus, even when smaller doses of SCH-23390 induce stronger unspecific activity than larger ones, with regard to the blockade of apomorphine-induced pecking, this drug seems to follow a direct dose-dependent response relationship. Other authors have also reported that apomorphine- and cocaine-induced behaviours (pecking and locomotor activity) were blocked by SCH-23390 in a dose-dependent way (De Vries et al. 1998, Zarrindast et al.1992).

The unspecific effects of the glutamatergic antagonist MK-801 (0.1, 1 and 2 mg/kg) were evaluated in experiment 14. Five parameters were taken as descriptive of pigeons' general behaviour and motility: head movements, wings movements, legs movements, stability when walking and effects on vision. It was observed that the larger MK-801 doses,

1.0 and 2.0 mg/kg, both produced strong dyskinesia, since the pigeons evidenced uncoordinated movements of the head, wings and legs and a general instability of their walking behaviour. The largest dose (2 mg/kg) also produced vision impairment. Conversely, the smallest dose (0.1 mg/kg) did not produce any of these effects. Even twenty-five minutes after injection, the effects of the two largest doses were clearly observable. Between two and a half and three hours after injection, all effects disappeared.

The previous mentioned Godoy study (Godoy 2000) evaluated the effects of haloperidol on the spontaneous pecking of pigeons. However, in that study the effects of haloperidol were tested on spontaneous pecking, which is more insensitive to a particular drug treatment. As a consequence, subtle differences in the level of pecking remain obscure. A different approach was followed here. Drug-free pecking was stimulated in pigeons by mildle food deprivation. The number of pecks for food elicited by saline-treated pigeons during the experimental sessions was considered a baseline, which was compared to pecking under a certain drug effect. Experiments 9 and 12 demonstrated that haloperidol (0.15 and 0.25 mg/kg) and SCH-23390 (3 µg/µl, 1µl per hemisphere) respectively, did not modify the pecking baseline. The same methodology was used in experiment 15 to evaluate the effects of the glutamatergic NMDA receptor selective antagonist MK-801 (80 and 120 µg/kg). These doses also did not affect the pecking baseline.

A first conclusion arising from the latter results is that the tested doses of the dopaminergic and glutamatergic antagonists did not affect the pecking behaviour *per se*. Another valuable conclusion is that they did not exert any observable effect on hunger levels. It could be speculated that if the dopaminergic agonist apomorphine has anorexic effects on pigeons (Deviche 1984), haloperidol and SCH-23390, being dopaminergic antagonists, might exert opposite effects and in so doing increase hunger. In principle, the doses tested in experiments 9 and 11, did not seem to have such an effect, since they did not affect pecking rates, a variable that could be affected by hunger. The glutamatergic antagonist MK-801 was not expected to affect hunger, and results of experiment 15 confirmed this. However, to properly assess whether a certain drug affects the hunger levels, it would be necessary to compare the amount of food that saline-treated and drug-treated pigeons eat. This question was not within the narrow scope of this thesis. Independent of whether they affect the level of hunger or not, it can be confirmed that haloperidol, SCH-23390 and MK-801 affected the number of pecks per session (in experiments, 6, 7, 10, 13, 16 and 17) as the relevant variable in the learning experiments.

Hyper-sensitisation of apomorphine-induced pecking

It is well documented that chronic neuroleptic treatment creates dopamine receptor hyper-sensitivity in the striatum (Kamer et al. 1981, Davis et al. 1978, Hitri et al. 1978, Burt et al. 1977, Muller & Seeman 1977). Kamer and colleagues (1981) demonstrated that a previous chronic treatment with haloperidol (for 21 days) resulted in a 52% increase in [^{3}H]-dopamine binding in the septum. These results suggest that haloperidol induces an up-regulation of the dopaminergic receptors. Behavioural dopamine hyper-sensitisation after a chronic haloperidol treatment was also demonstrated in rats. The locomotor activity measured in animals that received a pre-treatment of 4 weeks haloperidol and, after a week, a dopamine administration directly into the nucleus accumbens, was higher than in rats pre-treated with saline (Davis et al. 1978). The mechanisms by which haloperidol exerts the hyper-sensitisation effect still remain unclear. However, D2-like receptors regulate the dopamine release (Cubeddu et al. 1990, Dwoskin & Zahniser 1986) that could modify the dopaminergic synapses.

It has been determined that D2-like receptor antagonists enhance dopamine release by examining rat striatal slices (Dwoskin & Zahniser 1986) while apomorphine, a dopamine receptor agonist, reduces dopamine release by about 90% (Parker & Cubeddu 1985). A mechanism involving the regulation of dopamine release could be responsible for dopamine up-regulation as well. Additionally, D2-like receptors in the pre-synaptic membrane are known to reduce the release of dopamine in response to a depolarising stimulus (Cubeddu et al. 1990, Dwoskin & Zahniser 1986). It has been suggested that dopamine pre-synaptic receptor sub-sensitivity might contribute to psycho-stimulant-induced behavioural sensitisation (Pierce et al. 1995, Muller and Seeman 1978). Thus, it seems that the sub-sensitivity to pre-synaptic dopamine receptors as well as the increment of the dopamine release due to the action of D2-like receptors in the pre-synaptic membrane, play an indispensable role in the hyper-sensitisation phenomena induced by haloperidol.

In contrast to the above mentioned haloperidol results and the results of previous research using a D1-like dopamine antagonist SCH-23390, it was found here that SCH-23390 did not induce any behavioural hyper-sensitisation effect due to apomorphine. Previous studies showed that apomorphine-induced stereotyped behaviour increased in SCH-23390 hyper-sensitive rats (Dall'Olio et al. 1988, Gandolfi et al. 1988a, 1988b). However, these authors failed to find any increment in the SKF-38393-induced stereotyped behaviour (SKF-32393 is a selective D1-like receptor agonist) in SCH-23390 hyper-sensitive rats. Thus, Gandolfi and co-workers concluded that the expression of D1-like receptor hyper-

sensitisation was dependent on the balanced stimulation of the dopaminergic synapses such as through apomorphine (Dall'Olio et al. 1988, Gandolfi et al. 1988a, 1988b). The differences between the results presented here and those of Dall'Olio and Gandolfi and colleagues could be explained based on the way drugs were administrated. Pigeons in this study received the D1-like receptor agonist directly in the caudal striatum, while rats from previous studies received the same drug intraperitoneally and therefore affected the whole brain. The procedural differences as well as the species involved might near different metabolism rates, and consequently, different drug half-life times in both animal species and thus explain the contradictory results.

The hyper-sensitisation to apomorphine-induced stereotyped behaviuor produced by the NMDA antagonist MK-801 has some similarities to those produced by haloperidol. As demonstrated, both drugs produce hyper-sensitisation of the dopaminergic-induced stereotyped behaviours (Asin et al. 1996, Davis et al. 1978) as well as to increase the dopamine release at striatal levels (Sitges et al. 2000, Dwoskin & Zahniser 1986). However, MK-801 was found to increase the dopamine release at striatal levels, acting on the ventral tegmental area (Sitges et al. 2000, Mathe et al. 1999) and at striatal-limbic levels, acting through indirect mechanisms involving GABAergic neurones and/or sigma receptors (Ault & Werling 1999, Yoshida et al. 1998). The general mechanism underlying the hyper-sensitisation effects of apomorphine still remains unclear. It is not well understood whether this mechanism depends on the increase of dopamine release. Nevertheless, it seems to be the case that the regulation of dopamine release plays an important role in hyper-sensitisation to apomorphine. Additionally, chronic low MK-801 doses were found to induce response sensitisation when challenged with a higher MK-801 dose. Moreover, the sensitisation effect of MK-801 was enhanced when the challenge was induced with morphine (Jeziorski et al. 1994). Thus, it seems that the repeated administration of NMDA antagonists, which does not exert any behavioural effect, can produce long-lasting effects on neuronal responsiveness, as demonstrated in experiment 17. These effects, in turn, are intricately related to the effects of psychomotor stimulants (Wolf 1998).

It is well known that a pre-treatment administration of MK-801 prevents apomorphine sensitisation (Druhan et al. 1993). This was again demonstrated in experiment 16 of this work. However, the changes produced by MK-801 treatment on the NMDA and dopaminergic synapses could lead to the expression of the hyper-sensitisation of the apomorphine-induced response. Thus, when in the sixth session of experiment 16 MK-801 was suspended, a few sessions later, the blocked apomorphine-induced response showed a

constant increment. In experiment 17, in which a pre-treatment with MK-801 was carried out, as well as a treatment with apomorphine (leading to a normal sensitisation response), the results in test (under saline injection effect) showed a hyper-sensitised pecking behaviour. But, in contrast to experiment 16, this response was a decreasing one. The difference between both experiments was the time elapsed between the last MK-801 and first saline administrations. In between, apomorphine was also administrated (simultaneously or consecutively). Muller and Seeman proposed that the length of time needed for the reversal of dopamine hyper-sensitisation appears to be correlated with the duration of neuroleptic administration, regardless of the type and dose of neuroleptic employed, or of the kind of hyper-sensitive property monitored (Muller & Seeman 1978). Finally, the results of experiments 16 and 17 demonstrated that the MK-801 hyper-sensitisation effect appears two days after the last drug administration and lasts for about 9-10 days. In experiment 16, the hyper-sensitisation to apomorphine produced by six daily MK-801 administrations, appeared after the second day, when drug administration was cut off. Experiment 17 showed the end of this hyper-sensitisation effect to be after 7 to 9 days, during which time the hyper-sensitised pecking response constantly decreased. Thus, a maximal hyper-sensitised pecking response can be expected to take place between the third and seventh session, after six daily MK-801 administrations.

Dopamine and learning

Both dopaminergic antagonists haloperidol and SCH-23390, blocked the acquisition of an apomorphine-context association. In the case of haloperidol, the lower doses tested were more effective than larger ones in impairing the apomorphine-pecking acquisition and effects. However, as already mentioned, hyper-sensitisation of the dopamine receptors due to the higher haloperidol dose could explain this response increment. In the case of SCH-23390, larger doses were more effective. None of these drugs affected the expression of the already acquired association between apomorphine effects and context.

Pigeons injected with either low and high haloperidol doses, plus 0.5 mg/kg apomorphine (experiment 10) pecked less than pigeons injected only with apomorphine, but more than pigeons injected with saline. This outcome indicates that both haloperidol doses only partially impair the apomorphine-induced effects. During test with saline, pigeons trained with a low haloperidol dose plus apomorphine pecked significantly less than pigeons trained with apomorphine alone. Such an outcome suggests that the training with this

haloperidol dose produced a blockade of the association between apomorphine effects and context. On the other hand, test responses of pigeons trained with a high haloperidol dose plus apomorphine were approximately at the same level as the responses (actually, it was slightly higher) of pigeons treated only with apomorphine during the training phase. These findings indicate that even when the high haloperidol dose blocked the apomorphine-induced pecking during training, it simultaneously induced an hyper-sensitivity of dopamine receptors. This phenomenon probably masked the effectiveness of the dose in question in preventing the association between the apomorphine effects and contextual cues. As discussed before, it remains unclear which mechanisms underlie the hyper-sensitisation effect of haloperidol. But it was suggested that the dopamine pre-synaptic receptors' sub-sensitivity (only D2-like receptors are found pre-synapticaly) might contribute to psycho-stimulant-induced behavioural sensitisation by reducing releases of dopamine to a depolarising stimulus (Pierce et al. 1995, Cubeddu et al. 1990, Dwoskin & Zahniser 1986).

Thus, the results of experiment 10 suggest that the smaller haloperidol dose is more effective than the larger one in blocking the pecking effects of apomorphine, as well as in preventing the association between such effects and the context. This could be explained by the fact the low haloperidol dose was insufficient to induce the hyper-sensitisation phenomena induced by the high dose. Therefore, the blocking effect of the lower haloperidol dose was not masked by collateral effects of the drug on the receptors. Since the apomorphine effects constitute the US in this paradigm, haloperidol doses that seem to be more effective in blocking the US are at the same time more effective in impairing the acquisition of the apomorphine-context association. Godoy (2000) reported that an intermediate dose, 0.30 mg/kg haloperidol, impaired the apomorphine induced pecking effects (although it did not completely block them) and the apomorphine-context association (pigeons trained with 0.30 mg/kg haloperidol plus apomorphine responded at saline-trained control levels during test). Although her design presented some differences, on the basis of her results, it could be predicted, that a turning point in the dose-response relationship of haloperidol in relation to apomorphine-pecking hyper-sensitisation and learning will be located between 0.20 and 0.35 mg/kg haloperidol doses (the doses used in experiment 10). Nevertheless, to test this hypothesis several haloperidol doses within this range should be employed.

When the selective D1-like receptor blocker SCH-23390 was co-administered with apomorphine, it effectively blocked the apomorphine effects in an apparently direct dose-dependent way. The smallest dose tested (1µg/µl, in experiment 11 and 13), was almost negligible in blocking apomorphine pecking effects. Conversely, the largest tested dose

(5µg/µl, experiment 11), blocked them almost completely. Additionally, the intermediate dose (3µg/µl, in experiment 13), exerted an intermediate blocking effect. Surprisingly, the smallest dose, with its low impairing effects on UR pecking, was enough to impair acquisition of the apomorphine-context association. Unfortunately, the performance of an adequate statistical analysis was not possible due to the low number of subjects in the other groups. Furthermore, the intermediate dose (3µg/µl, SCH group) impaired the acquisition of the association. These findings suggest that SCH-23390 impairs the acquisition of the apomorphine-context association in a dose dependent manner. However, once this association has been acquired, the effective 3µg/µl SCH-23390 dose does not affect its expression, as demonstrated in experiment 13.

Thus, D1-like and D2-like dopamine receptors seem to be involved in the apomorphine-context association. Blockade of one or the other with effective doses of corresponding selective antagonists impaired the magnitude of the UR pecking and consequently, the conditioning of the apomorphine effects to the context, as expressed by the magnitude of CR pecking. None of these antagonists seemed to block the expression of the already acquired associative learning. These conclusions are in line with former results (Godoy 2000), which also suggest that the acquisition process in this conditioning paradigm is mediated by dopaminergic mechanisms, while its expression remains independent of this system.

Actually, the role of the D1-like and D2-like receptors in dopamine-mediated conditioning is not yet well understood. Studies on pigeons are rather scarce and most reports refer to amphetamine or cocaine-induced conditioning in other species. With the exception of some studies already mentioned (Godoy 2000, Godoy & Delius 1999, Burg et al. 1989), among them, many other studies using avian subjects dealt with the role of dopamine receptors in behavioural responses, but not in learning mediated by dopaminergic drugs. However, there are some reports on mammals concerning the role of each receptor type in learning. Carey (1990), found in unilaterally 6-OHDA lesioned rats, that SCH-23390 and haloperidol partially blocked the spontaneous unconditioned response induced by apomorphine while both drugs combined blocked it completely. However, none of the above mentioned substances blocked the expression of the already acquired CR in a group previously trained in a distinctive environment with repeated apomorphine injections. Results on amphetamine- or methamphetamine-induced conditioning are sometimes contradictory. Some authors found that acquisition was blocked by the D1-like receptor antagonist SCH-23390, others by the D2-like receptor antagonists YM 09151-2 and still other ones by

pimozide (Hamamura et al. 1991, Ujike et al. 1989, Beninger & Hahn 1983). Other authors also reported that acquisition was blocked by SCH-23390 but not by pimozide, or by the also D2-like receptor antagonists metoclopramide or Ro 22-2586 (Mazurski & Beninger 1991, Drew & Glick 1990). The acquisition of an amphetamine-induced conditioned place preference was found to be blocked by SCH-23390 and metoclopramide, only the first one blocking its expression (Hiroi & White 1991, Hoffman & Beninger 1989). The development of sensitisation to the rewarding effects of cocaine was prevented by SCH-23390 but not by the D2-like receptor antagonist raclopride (Shippenberg & Heidbreder 1995). As far as a comparison of different apomorphine- amphetamine- and cocaine-induced conditioned responses is possible, it seems from the results of these studies that intact D1-like receptors are necessary for conditioning to dopaminergic drugs, while the role of D2-like receptors remains unclear. This issue deserves more investigation using other receptor-specific drugs, which may be of value for the discrimination of the functions of each receptor type in conditioning.

All the above cited reports are in line with the results of experiments 10 and 13 in that dopaminergic antagonists affect acquisition but not expression of conditioning to the effects of dopaminergic agonists. This finding suggests that during acquisition, dopaminergic receptors mediate some brain plasticity, which is not dopaminergic and would influence subsequent behaviour. Thus, changes in behaviour as a consequence of learning can be later expressed even through the blocked dopaminergic systems. The nature of the neurotransmitter system supporting the expression of dopamine-mediated conditioning is not known, but a close interaction between dopamine and glutamate in relation to conditioning processes (e.g. Amalric et al. 1994) makes the glutamatergic system a good candidate. For this reason, the effect on conditioning of MK-801, an antagonist selective for glutamatergic receptors of the NMDA type, was tested using apomorphine-context conditioning.

Glutamate and learning

The glutamatergic antagonist MK-801, selective for the ion channel associated with NMDA glutamate receptors, seemed to block the acquisition of the association between apomorphine effects and contextual cues, as well as the expression of the already established association, as shown by the results of experiments 16 and 17.

In experiment 16, co-administration of MK-801 during training effectively reduced pecking responses induced by 0.5 mg/kg apomorphine, in a clear dose-dependent manner,

with 50µg/kg MK-801 exerting the weakest effect and 120µg/kg MK-801 the strongest one. The intermediate dose, 80µg/kg MK-801 exerted an intermediate effect. This range of MK-801 doses was shown to have no *per se* effects on general motility or on drug-free pecking, as shown in experiments 14 and 15. These results demonstrated that this glutamatergic antagonist was effective in blocking at least some effects of the dopaminergic agonist apomorphine. Since apomorphine effects constituted the US in the apomorphine-context association, the impairing effects of MK-801 consequently affected acquisition, as confirmed by results of the first test session. However, the effects on acquisition were not so clearly dose-dependent, because pigeons trained with 80µg/kg MK-801 plus apomorphine exhibited lower CR pecking than pigeons trained with 120µg/kg plus apomorphine, in the first test session with saline. A surprising result was that independent of the dose being used, responses of all MK-801 trained groups increased over the repeated test sessions with saline, and by the end of test they were higher than those of the control group Ref-Apo. During test, the smallest MK-801 dose induced the strongest response increment.

In experiment 17 the effects of MK-801 on the expression of the already acquired apomorphine-context association were tested. Until their responses reached an asymptotic level, pigeons trained with repeated apomorphine injections responded less in a subsequent test with injections of 100 µg/kg MK-801 than pigeons equivalently trained with apomorphine and tested with saline. This later outcome suggests that 100 µg/kg of glutamatergic antagonist MK-801 were effective in blocking the expression of an already acquired association between effects of the dopaminergic apomorphine and the experimental contextual cues.

In experiment 17, effects of MK-801 on acquisition were also tested through injection in a pre-training phase, before the training with repeated apomorphine administration. It was found that previous treatment with repeated injections of 100 µg/kg MK-801 did not affect the course of sensitisation. Pigeons treated in that way exhibited a normal pecking sensitisation curve in response to apomorphine just like pigeons without previous drug treatment. However, during subsequent tests with saline, pigeons pre-treated with MK-801 before of the apomorphine training, exhibited higher responses than control pigeons, which only received a training under apomorphine. Nevertheless, these differences were not significant.

With reference to test responses of pigeons treated with simultaneously or sequentially repeated injections of MK-801 and apomorphine, two unexpected and apparently related results were obtained (experiments 16 and 17). In experiment 16, where pigeons were trained for six daily sessions with 0.5 mg/kg apomorphine simultaneously injected with one of the MK-801 doses (50, 80 or 120 µg/kg), and then tested with saline, their responses increased

over test sessions, instead of decreasing as expected. In experiment 17, when pigeons treated with six daily injections of 100 µg/kg MK-801 followed by six daily injections of 0.5 mg/kg apomorphine were tested with saline, their responses were higher than those of pigeons treated only with apomorphine in an equivalent training design. Pigeons trained with apomorphine and tested with MK-801 did not display any sensitisation of test responses. A treatment with MK-801 before or during a training with apomorphine produced an hyper-sensitisation of the apomorphine-induced pecking response. This hyper-sensitisation phenomena produced by the blockade of NMDA receptors was discussed above. However, it is of value to point out that MK-801 did not affect the general motility (as shown in experiment 14) nor the drug-free pecking behaviour: 80 and 120µg/kg MK-801 did not produce an increment in drug-free pecking for food in experiment 15. Thus, the putative hyper-sensitisation induced by MK-801 seems to be exerted only on pecking elicited by apomorphine. Such sensitisation was observed during test with saline, i.e. when the conditioned pecking response was expressed. It is tempting to speculate that MK-801 sensitises the CR acting on learning mechanisms. As mentioned before, the results obtained with the apomorphine-context paradigm indicate that apomorphine acts through dopaminergic receptors to induce changes in the nervous system. These changes are not dopaminergic in nature and are responsible for the subsequent learning-dependent behaviour modification. As hypothesised earlier, such a change could be glutamatergic in nature due to the apparently close relationship between the dopaminergic and glutamatergic systems in learning. The observed sensitisation produced by MK-801 on CR pecking, would be in line with such an hypothesis.

Dopamine-glutamate interaction

Several results obtained in the experiments presented in this dissertation evidence interaction between the dopaminergic and glutamatergic systems in learning. In experiment 16, the co-administration of MK-801 impaired the development of apomorphine-induced pecking sensitisation during training. Several examples of experimental evidence (e.g. Godoy & Delius 1999, Wynne & Delius 1995, Lindenblatt & Delius 1987) indicated that the sensitisation increment was due to the development of conditioned pecking (CR) elicited by the experimental context (CS), which added to the unconditioned pecking (UR) directly elicited by apomorphine (US). Thus, the finding that the glutamatergic antagonist MK-801 impaired pecking response conditioning induced by the dopaminergic apomorphine, suggests

that the development of a classical conditioning association between apomorphine effects and the context actually requires both the dopaminergic and the glutamatergic systems. The first pecking response to apomorphine was assumed to represent the UR pecking (although some CR pecking was also developed in the first session).

The fact that co-administration of MK-801 did not reduce pecking below the level of the first response to apomorphine, but instead, reduced the subsequent sensitisation, indicates that this glutamatergic antagonist did not interfere directly with mechanisms underlying pecking but rather on mechanisms underlying learning. The finding in experiment 17, in which MK-801 impaired the expression of an already-acquired association between apomorphine effects and context, constitutes further evidence for the glutamatergic-dopaminergic-system interactions in this kind of learning. The expression of dopamine-mediated learning was not possible if the glutamatergic system was blocked. However, experiments 14 and 15 indicate that MK-801, in the doses tested here, does not effect motor responses. Therefore, its impairing effects on the development of sensitisation and on the expression of the already acquired conditioning cannot be explained as MK-801 affecting the performance of the CR, but rather and more probable as affecting the CS.

Additionally, the finding of experiments 16 and 17, that a previous or a simultaneous treatment with MK-801 induces increments in conditioned pecking developed with an apomorphine treatment, also indicates that the glutamatergic and the dopaminergic systems interacted (although mechanisms such as those of MK-801 mediated sensitisation are still not fully understood). Furthermore, possible interactions between dopamine and glutamate suggested here will be illustrated and further discussed in terms of a model developed by Wickens (1990) on the role of both neurotransmitters in the striatum during the learning process.

Evidence of such interaction between both neurotransmitter systems in learning has also been reported for mammals. MK-801 has been found to block the development of behavioural sensitisation induced by apomorphine and of conditioned place preference, induced by methamphetamine in mice (Kim & Jang 1997, Druhan et al. 1993). Antagonists of the glutamatergic receptors of types NMDA and AMPA, as well as antagonists of the dopaminergic receptors D1 and D2, have been found to influence the acquisition and expression of cocaine-induced conditioning (Druhan & Wilent 1999, Cervo & Samanin 1996, 1995, Kim et al. 1996).

Apomorphine-context paradigm and the Wicken's learning model

The results of the experiments presented in chapter VII suggest that a possible Hebbian' post-synaptic interaction takes place in the apomorphine-induced learning paradigm. According to Wickens' model of associative learning, glutamatergic inputs to striatal cells are selectively potentiated by a dopaminergic input on the same cells (Figure 8.1). It seems adequate to conclude that apomorphine acts on post-synaptic membranes of striatal cells as an US, while glutamatergic synapses act on the same post-synaptic cell as a CS. The interaction of these few synapses becomes potentiated, resulting in the expression of the CR. The US signal (apomorphine/dopamine) selects some few glutamatergic synapses. Such selection is possible only by the simultaneous activation of both synapses. The dopaminergic income on striatal cells is activated by apomorphine acting directly on dopaminergic receptors. Simultaneously, the only glutamatergic synapses activated at this time are those ones carrying the relevant contextual information. It is worthwhile to mention that when the contextual environment changes, this selection does not take place. Keller performed an experiment in which pigeons were injected with apomorphine during four daily sessions, placing the animals in different experimental cages each time (Keller & Delius 2001). She found that pigeons showed a stereotyped pecking response from the first to the fourth sessions. However, this pecking response was not sensitised and therefore remained nears constant. This finding strongly supports the previously mentioned hypothesis of selection of glutamatergic input by means of dopaminergic activation. If there is no clear CS signal, then there is no CS-glutamatergic connection selected and therefore no CR expression. According to the "conditional hypothesis" mentioned in chapter III, increments of responses during a given sensitisation phenomena have an important if not exclusive learning component

Experiment 2, in which long-term retention of the apomorphine-context was assessed after an interval of about two years also supports the hypothesis that the selection and enhancement of a given connection can be long-lasting (years). It is hard to imagine such a change in biochemical mechanisms that lasts that long without thinking of learning mechanisms as well. The "conditioning hypothesis" seems to be more probable than the pharmacological ones. Even though experiment 3 (in which the dopaminergic receptors were measured after chronic apomorphine administration) demonstrates that this apomorphine treatment induces increments of D1-like receptor amounts and decrements of D2-like ones in the basal telencephalon. These changes in receptor concentrations may be due to learning as well as pharmacological processes. A possible experimental design assessing this controversy

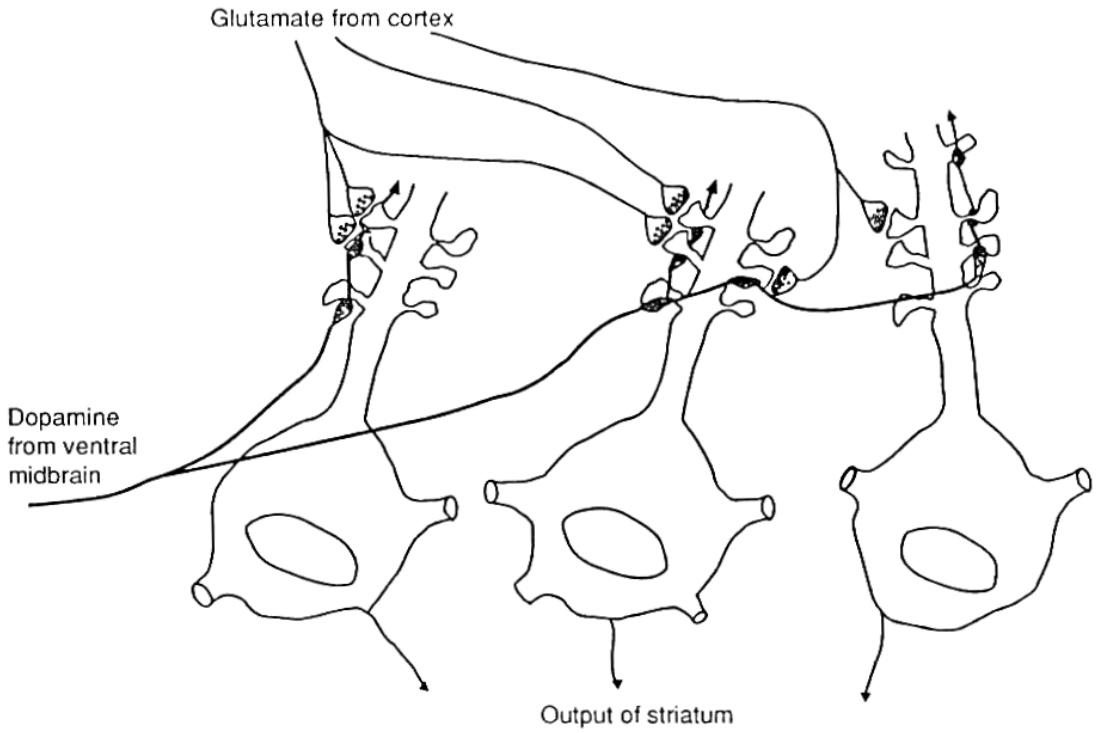


Figure 8.1 Scheme of the glutamatergic and dopaminergic interaction in the striatum. Striatal medium spiny neurones receive glutamatergic afferent from the cortex, making axospinous contact with dendritic spines that also receive dopaminergic synapses from the ventral midbrain. Most of the glutamatergic input to the striatum is thought to be sensory in nature, bringing to the striatum representations of events in the environment. The striatal output is thought to be motor in nature, so the striatum is viewed as a sensory-motor interface in the brain (taken from Beninger 1993).

might be a group of pigeons receiving apomorphine injections, immediately placed in a different contextual environment for every session as Keller and Delius did (2001). A second group of animals could also receive apomorphine, but then be placed consistently in the same contextual environment. Pecking sensitisation does not develop when pigeons are placed in a variety of different environments. Consequently, it should be expected that this group will not show changes in basal ganglia dopamine receptor concentrations when the binding assay technique is performed. Opposite results should be expected for the group trained in a constant context. Here, increases in D1- and decrease in D2-like receptor concentrations are expected.

A simultaneous visual discrimination was designed for the comparison of results of dopaminergic and glutamatergic receptor antagonists with those of apomorphine-induced learning paradigm experiments involving drug-free associative learning. This paradigm called

“œloured grit-food association” was developed in an attempt to have a fast and reliable learning paradigm, in which antagonist drugs can be employed. The paradigm itself proved to be powerful and sensitive enough to detect changes in responses measured by the index created for this purpose. It was demonstrated that a latent inhibition procedure on acquisition of the positive stimulus-food association effects a decrement of the choice response (measured by the discrimination index). In addition, it was demonstrated that the effect of a second procedure impaired the acquisition of new learning when the same stimuli were involved. This second procedure was a reversal learning task, in which one of the negative stimulus becomes positive, and the previous positive stimulus becomes negative. This reversal learning procedure also decreases responses (measured by the discrimination index) in similar ways, as a latent inhibition procedure. These findings are in line with evidence of previous work in latent inhibition and even in reversal learning procedures (Schauz & Koch 1998, Killcross & Balleine 1996, Delius et al. 1995, Nakagawa 1992).

Even though the coloured grit-food association proved to be reliable as an associative learning paradigm, it failed as a useful tool for the assessment of dopaminergic and glutamatergic involvement in such a learning process. These results suggest that the neuronal mechanisms underlying this kind of learning may be largely different from those proposed by Wickens. However, the effect of the procedure (i.e. injections) on arousal levels should be further tested. This can affect cognitive responses, thus masking the effects of the drugs on the discriminative task. A possible modification of the paradigm which could resolve this masking problem could consist of reducing the cognitive activation of the discriminative task. This could be achieved by reducing the number of stimuli from six to two, and rewarding one stimulus out of two, unlike the present work in which one out of six held a reward.

Apomorphine vs. simultaneous discrimination paradigm

Figure 8.2 illustrates schematically both learning procedure used in the present study. The apomorphine-induced learning can be interpreted as a particular case of the Wickens’ model (Figure 8.2a). According to this model, the apomorphine administration acts mainly as an US through the reward system (the mesolimbic pathway), and the contextual cues (CS) acts on the striatal cells through the glutamatergic cortical input which is at the same time the outcome of the visual information processing. The US-CS association develops in striatal cells which in turns act as a sensory-motor interface yielding the pecking response (CR) as output. The results of the experiments blocking the dopaminergic and glutamatergic receptors

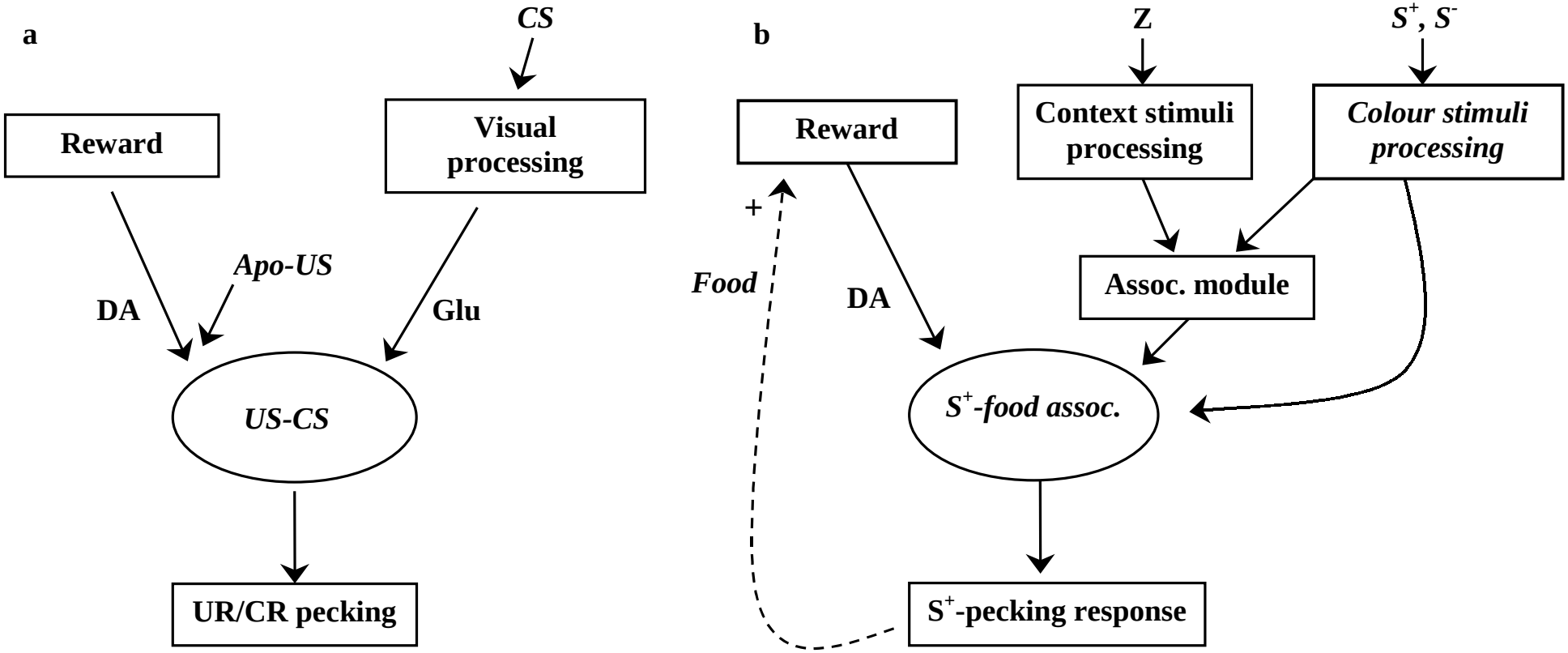


Figure 8.2. Schematic diagrams that summarise the apomorphine and simultaneous colour discrimination paradigms. **a)** Apomorphine-induced learning. According to Wickens' learning model, a dopaminergic input on striatal cells modulates the glutamatergic cortical input. The outcome of this modulation is the selection and enhancement of a particular CS-CR connection. **b)** Simultaneous colour discrimination. It involves an additional associative module. The S^+ could be associated to the reward food by itself and through a configural association (by the way of the associative module) at the same time. This context module may process the stimuli (Z) that arise from the exploratory and searching behaviour together with the contextual cues of the experimental situation. These Z stimuli may at the same time be associated with the S+ and together play the role of a compound stimulus. Therefore, the S^+ could be associated with food by acting directly as a single S^+ or through the associative module (for more details see main text).

on apomorphine-induced learning are in full agreement with this scheme. Wickens claims that dopamine plays an important role selecting and enhancing the glutamatergic synapses involved in relating the visual information of a particular CS and a particular CR. Glutamate is necessary for the acquisition as well as for the retrieval of such association. The predictions of this model were generally supported by the results of chapter VII.

However, the pharmacological experiments performed with the simultaneous colour discrimination (chapter VI) failed to agree with the predictions of this model. To explain the results of the pharmacological experiments on the simultaneous colour discrimination the scheme of the figure 8.2b was developed. This learning scheme has some similarities with the apomorphine one. Both learning processes require a reward factor as US, one mediated by apomorphine stimulation and the other by the ingestion of food, also both involve the output of the visual information processing as carrier of the CS or the S^+ information to the brain area in which the associations between US-CS or S^+-food are take place. Differently from the apomorphine scheme, the simultaneous colour discrimination involves an associative module that was conceived based on the model for a multiple response system proposed by Schmajuck-Lamoreux-Holland (SLH). According to this model, the conditioned stimuli can behave as a simple conditioned stimuli or as an occasion setters (Lamoreux et al. 1998). The SLH model assumes that a stimulus acts as a simple conditioned stimulus when it acts on a response system through its direct excitatory or inhibitory associations and as an occasion setter when it acts on a response system through its configural associations (by way of the associative module).

Therefore, representations of simple and configural conditioned stimuli compete to gain association with the unconditioned stimulus. The conditioned stimulus can act both at a simple CS and an occasion setter at the same time. Although the SLH model describes a classical conditioning learning procedure, the term occasion setting was originally introduced by Skinner (1938) to refer the relationship between a discriminative stimulus and the instrumental responding that was reinforced in its presence (Bonardi 1998). Moreover, Bonardi argues that there is a direct operational analogy between an occasion setter in whose presence a CS-US relationship holds and a discriminative stimulus in whose presence a response-reward contingency operates (Bonardi 1998). In the recent decades this parallel between Pavlovian and instrumental conditioning has received increased support through the suggestion that both kinds of association processes arise from the same learning mechanism (Mackintosh & Dickinson 1979).

The apomorphine-induced learning was demonstrated to be NMDA-dependent (experiments 16 and 17) during both acquisition and retrieval. However, the simultaneous colour discrimination paradigm was not affected by the blockade of NMDA receptors. The direct action of the S^+ or through the proposed associative module on the S^+-food association seem to be NMDA-independent. Despite of the fact that visual information processing seems to require glutamatergic activity, it is not well understood how the visual colour information is processed in the bird's brain (for review see Güntürkün 1996). Thus, it may be that NMDA receptors do not play any role in the processing of visual colour information. Administration of the NMDA antagonist MK-801 did not affect the visual colour processing as well as the learning of the association of a given colour with food. It could be then inferred that the learning of the simultaneous colour discrimination is NMDA-independent as demonstrated in experiment 8 . Nevertheless, further experiments directed to asses the non-NMDA role in either the visual colour information processing and simultaneous colour discrimination are needed. These experiments will reveal whether the glutamatergic activity necessary for these processes involve or not other than NMDA glutamatergic receptors.

In summary, the associative learning model proposed by Wickens was only partially supported in this dissertation by the assessment of the dopaminergic and glutamatergic roles on two associative learning procedures. It was demonstrated that dopamine and glutamate play a crucial role in the apomorphine-induced learning paradigm. The coloured grit-food association seems to be based on different neuronal systems than those proposed by Wickens' model. Both learning paradigms share some characteristics; both employed rewarding stimuli that involve feeding behaviour and stimuli that involve processing of visual information. However, these two behaviourally related learning paradigms seem to be in fact different when the neuronal systems that could underlie these processes are examined. Further experiments are undoubtedly needed to better define the neuronal systems responsible for the coloured grit-food association.

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Lebenslauf

Name: Martín Javier Acerbo.

Geboren : 17.03.1969 in Pergamino, Argentinien.

Staatsangehörigkeit: argentinisch

Schulbildung und Studium

- 1975-1981 Grundschule im Escuela Nacional N°7 Formosa. Arrecifes, Argentinien.
- 1982-1986 Abitur im Colegio Nacional Normal Superior Enrique Udaondo, Arrecifes, Argentinien.
- 1987 Studiums-Vorbereitungskurs in der Universität Buenos Aires, Argentinien.
- 1988-1996 Diplom im Fach Biologie, Naturwissenschaftlichen Fakultät, Universität Buenos Aires, Argentinien.
- Seit Februar 1997 Doktorand in der Sozialwissenschaftlichen Fakultät, Allgemeine Psychologie. Betreuer: Prof. Dr. J.D. Delius, Universität Konstanz, Deutschland.

Forschungserfahrungen

- 1994-1995 Wissenschaftliche Hilfskraft, Universität Buenos Aires. Immunogenetik (Prof. F. Roissman), Medizinische Fakultät;Allgemeine Parasitologie (Prof. C. Wisnivesky-Colli), Biologie Fakultät.
- 1995-1996 Gaststudent an der Universität Konstanz, gleichzeitig Wissenschaftliche Hilfskraft, Biochemie und Pharmakologie (Dr. S. Ullig).
- 4/1996 Laboraufenthalt im Institut für Tier Biochemie und Genetik, Slowakische Wissenschaftliche Akademie, Ivanka pri Dunai Slowakische Republik (Prof. L. Kostal).
- 9/1998 und 6/1999 Laboraufenthalt im Biopsychologie Labor Bochum-Ruhr Universität, Bochum (Prof. Dr O. Güntürkün).
- 1996-1998 Wissenschaftliche Hilfskraft, Allgemeine Psychologie, Sozialwissenschaftliche Fakultät, Universität Konstanz. (Prof. Dr. J.D. Delius)

- 1998-2000 Graduierte Stipendiaten nach dem Landesgraduiertenförderungsgesetz, Universität Konstanz.
- 04/2000-08/2000 Einstellung als wissenschaftlicher Angestellter an der Universität Konsatnz (IIa BAT; Prof. Dr. J.D. Delius)
- 09/ bis 11/2000 Stipendiate nach dem Volskwagen Stiftung an der Universidad Nacional de Cuyo, Mendoza, Argentinien (Prof. Dr. Pascual Gargiulo).
- 12/2000-01/2001 Einstellung als wissenschaftlicher Angestellter an der Universität Konsatnz (IIa BAT; Prof. Dr. J.D. Delius)
- 03/2001-06/2001 Einstellung als wissenschaftlicher Angestellter an der Universität Konsatnz (IIa BAT; Prof. Dr. J.D. Delius)

Lehrerfhrungen

- 1999-2000 Hilfdozent im Fach Psychologie, Physiologische Psychologie, Transmittersysteme als Wirksubstrate für Psychopharmaka. (mit Dip S. Keller)
- 2001 Seminar im Fach Psychologie, Psychopharmacological manipulations of transmitter systems.

Tagungen und Publikationen

Roisman F., Izuel M., Fainboim H., Morelli A., Rubio A. & Acerbo M. (1993). Study of mononuclear cell infection by the Hepatitis B virus. Expociencia, Buenos Aires University.

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