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Identification of Genes Differentially Expressed upon Cocaine Treatment and of New Rat Semaphorins

THESE

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CONTENTS

ABBREVIATIONS:

ATII: angiotensin II
BG: basal ganglia
CART: cocaine- and amphetamine- regulated transcript
CCK: cholecystokinin
CRF: corticotropin-releasing factor
DA: dopamine
DAergic: dopaminergic
DARPP: DA- and cAMP-regulated phosphoprotein
Dyn: dynorphin
Enk: enkephalin
EP: entopeduncular nucleus
FRAs: Fos-related antigens
Glu: glutamate
Glu-ergic: glutamatergic
GP: globus pallidus
HPA axis: hypothalamic-pituitary-adrenal axis
5-HT: serotonin
IEG: immediate early genes
KO: knockout
NA: nor-adrenaline
NAcc: nucleus accumbens
NT-3: neurotrophin-3
OT: olfactory tubercle
PKA: protein kinase A
SN: substantia nigra
SNc: substantia nigra, pars compacta
SNl: substantia nigra, pars lateralis
SNr: substantia nigra, pars reticulata
SP: substance P
STN: subthalamic nucleus
TH: tyrosine hydroxylase
VP: vasopressin
VTA: ventral tegmental area

ABSTRACT

At the behavioral level drug addiction is defined as compulsion to take a drug with loss of control over apparently voluntary acts of drug seeking and drug taking. At the neuronal level drugs elicit two types of responses: neuronal adaptations, i.e. homeostatic responses to excessive stimulation, and synaptic plasticity. At the molecular level both processes are paralleled by changes in gene expression pattern in the mesolimbic dopaminergic system. The genes differentially expressed upon drug treatment include transcription factors, neurotransmitter and axon guidance cues. The detailed picture of gene expression changes is necessary in order to dissect the role of different molecules in the development of the distinct temporal and functional phases of drug addiction. Therefore, in the present study the screening for differentially expressed genes upon acute cocaine withdrawal was performed in three brain regions: the striatum, the tegmentum and the hippocampus. Two modifications of differential display of mRNA were introduced allowing first for increased reproducibility and easy handling of the method, and second for a gene-specific screening for the semaphorins, a family of axon guidance cues. The differentially expressed candidates displayed a high range of functional characteristics, illustrating the complexity and the variability of neuroplastic changes induced in the brain by psychostimulant treatment. In particular, CD81, a tetraspanin, was found to be overexpressed in the nucleus accumbens upon cocaine treatment. *In situ* hybridization studies allowed for localization of CD81 mRNA in the brain regions functionally related to the regulation of cardiovascular function and fluid homeostasis, most of them also expressing the neuropeptide galanin. In addition, CD81-deficient mice exhibited sex-dependent altered sensitivity to cocaine, as well as modified dopamine metabolism. One protein related to neurodegenerative processes in the brain, γ-synuclein, was found to be overexpressed in the tegmentum upon cocaine treatment, suggesting a link between the neuroplasticity changes related to neural degeneration and drug addiction. Finally, one new vertebrate member of the semaphorin family and five novel rat orthologues of class 3, 4 and 7 semaphorins have been identified, whereas the application of newly developed method of Enriched Differential Display allowed for finding of semaphorin differential regulation in various brain regions upon cocaine treatment.

RESUME

Au niveau comportmental la dépendance est définie comme un désir compulsif de prendre de la drogue avec perte de controle d’actes apparemment volontaires comme la recherche et la consommation de stupéfiants. Au niveau neuronal les stupéfiants provoquent deux types de réponses: des adaptations neuronales, c'est-à-dire des réponses homeostatiques à une stimulation excessive, et la plasticité synaptique. Au niveau moleculaire ces deux effets sont liés à des changements simultanés du mode d'expression de gènes dans le système dopaminergique mésolimbique. Les gènes différentiellement exprimés après traitement aux drogues, comprennent des facteurs de la transcription, des neurotransmetteurs, et des molecules de guidance axonale. Une image détaillée des changements d'expression genique est nécessaire pour disséquer le rôle des différentes molécules dans le développement des diverses phases temporelles et fonctionnelles associées à la dépendance. C'est pourquoi, dans l'étude présente, on a effectué la recherche des gènes différentiellement exprimés après traitement aigu à la cocaine et après retrait, dans trois régions du cerveau: le striatum, le tegmentum et l'hippocampe. Deux modifications de la méthode de display différentiel de mARN ont été introduites, permettant, premièrement, d'augmenter la reproductibilité et de faciliter le maniement de la méthode, et deuxièmement, d'effectuer une recherche spécifique de sémaphorines, une famille des molécules de guidance axonale. Les gènes exprimés différentiellement representent une large palette de candidats aux charactéristiques fonctionnelles diverses, illustrant la complexité et la variabilité des changements neuroplastiques induits par un traitement à un psychostimulant dans le cerveau. En particulier, nous avouns trouvé que le CD81, une tetraspanine, est surexprimé dans le noyau accumbens après treatment à la cocaine. Une étude par hybridisation *in situ* a permis de localizer le mARN de CD81 dans des régions du cerveau fonctionnellement liées à la régulation de la fonction cardiovasculaire et de l'homéostase des fluides. La plupart de ces régions expriment aussi le neuropeptide galanine. En plus, les souris déficientes pour le gène du CD81, démontraient des changements de la sensibilité à la cocaine, variables selon le sexe, ainsi que des modifications du métabolisme de la dopamine. En outre, nous avons montré qu’une proteine, liée aux processus neurodegeneratifs dans le cerveau, la γ-synucleine, était sur-exprimée dans le tegmentum après traitement à la cocaine, ce qui suggère un lien entre les changements neuroplastiques liées à la neurodégénérescence et la dépendance. Finalement, un nouveau membre de la famille des sémaphorines de vertebrés et cinq orthologues de sémaphorines des classes 3,4 et 7 chez le rat, ont été identifiés, alors que l'application de la nouvelle méthode du Display Différentiel Enrichi que nous avons developpé a permis de démontrer la régulation différentielle des sémaphorines dans diverses régions du cerveau après traitement à la cocaine.

I. INTRODUCTION

The present study was aimed at investigating molecular cues involved in the action of cocaine, one of the major drugs of abuse, and in the establishment of drug addiction. To achieve this purpose, the screening of differentially expressed genes was performed in the structures of the brain related to the dopaminergic (DAergic) system and basal ganglia (BG). Before the presentation and discussion of the results, a general introduction will be given to familiarize the reader with the subject.

1. *Dopamine*

DA is synthesized in the presynaptic terminal from tyrosine which is converted into L-DOPA with tyrosine hydroxylase (TH) (Figure 1), then into DA by aromatic amino acid decarboxylase. DA is then taken up and stored in synaptic vesicles in the presynaptic terminal. After the release of DA from the vesicles into the synaptic cleft, reuptake by the dopamine transporter (DAT) in the presynaptic membrane occurs after which the DA is transported back into vesicles or is degraded to DOPAC by type B monoamine oxidase (Olivier et al., 2000; Shih et al., 1999).

DAT is densely concentrated in the substantia nigra, pars compacta (SNc) and scattered throughout the ventral tegmental area (VTA) (Freed et al., 1995). Both regions contain high concentrations of DAT mRNA. The relative abundance of DAT mRNA varies across subregions: it is highest in SNc, medium in the parabrachial pigmentosis and lowest in the intrafascicular nucleus (Burchett and Bannon, 1997). Low levels of DAT mRNA are present in the central gray and hypothalamus. In addition, DAT mRNA can be detected in the amygdala, caudate-putamen, nucleus accumbens (NAcc), pons/medulla, globus pallidus (GP), pituitary and frontal cortex (Maggos et al., 1997).

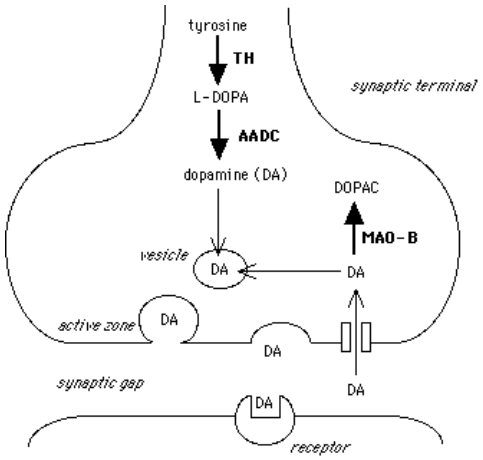


Figure 1. DAergic terminal with cycle of synthesis, storage, release and removal of DA. AADC, aromatic amino acid decarboxylase; MAO-B, type B monoamine oxidase; TH, tyrosine hydroxylase (adapted from (Cooper and Bloom, 1996))

DA receptors are G-protein-coupled seven transmembrane proteins and include two major classes, D1-type and D2-type. D1-type include D1 and D5 receptors (Emilien et al., 1999) and activate the adenylyl cyclase via a Gsα-subunit leading to an increase in cAMP concentration (Kebabian and Calne, 1979). One important pathway of D1 signaling includes DA- and cAMP-regulated phosphoprotein (DARPP)-32 phosphorylation by protein kinase A (PKA) which inhibits dephosphorylation of NMDA receptors via inhibition of an endogenous protein phosphatase-1, thus enhancing NMDA responses (Blank et al., 1997). It occurs synergistically with cAMP/PKA-dependent NR1 subunit phosphorylation and provides a link between DA signaling and synaptic plasticity (Greengard et al., 1999).

D2-type comprise D2, D3 and D4 receptors (Emilien et al., 1999) and inhibit the adenylyl cyclase via a Giα-subunit or are linked to the phosphatidylinositol turnover or to potassium and calcium channels (Vallar et al., 1988).

D1 receptors are abundant in the caudate-putamen, NAcc and olfactory tubercle (OT), as well as the cerebral cortex, limbic system, hypothalamus, thalamus (Mengod et al., 1989) and in striato-nigral axons in the substantia nigra, pars reticulata (SNr) (Ariano et al., 1989). Activation of D1 receptors increases immediate early genes (IEG) expression in the striatum and conductance through the NMDA receptor. c-fos, but not zif268, induction is at least partially mediated by NMDA receptor activity (Keefe and Gerfen, 1996). Despite the opposite action of D1 and D2 receptors on adenylyl cyclase activity, some D1 effects depend on the synergistic action of D2 receptor (LaHoste et al., 2000). For example, the expression of zif268 is reduced by application of a D2 receptor agonist, whereas the simultaneous application of D1 and D2 receptor agonists increases the induction of zif268 compared to D1 receptor agonist alone (Keefe and Gerfen, 1995). This synergistic action results from the interaction between different neurons, since D1 and D2 receptors are normally not expressed in the same cells (Gerfen et al., 1995). It plays an important role in the case of drug self-administration, where D2 receptors mediate the reinforcement of the stimulant drug and D1 receptors play a permissive role (Missale et al., 1998).

D2 receptors are highly expressed postsynaptically in the caudate-putamen, NAcc and OT (Mengod et al., 1989) and as autoreceptors on DAergic dendrites in the medial two thirds of the SNc, with lesser density in the dorsal VTA (Akaoka et al., 1992).

D3 receptors are expressed in the OT, NAcc, the islands of Calleja, the hypothalamus and as autoreceptors in DAergic cells laterally in the SN-VTA (Bouthenet et al., 1991). D3 receptors are localized preferentially in limbic brain areas and affect locomotion and perhaps reinforcement and reward (Shafer and Levant, 1998).

D4 receptors are present in the frontal cortex, midbrain, amygdala, and medulla (Van Tol et al., 1991). Their affinity is not limited to DA, but expands also to adrenaline and noradrenaline, explaining their role in coordinating signaling between the different catecholamine neurotransmitter systems (Hartman and Lanau, 1997; Lanau et al., 1997).
D5 receptors are expressed in the hippocampus, hypothalamus and pretectal nucleus (Ciliax et al., 2000; Meador-Woodruff et al., 1992). Remarkably, DA is 10-fold more potent at the D5 receptor than at the D1 receptor.
Among all types of DA receptors only D2 and D3 subtypes are expressed presynaptically. The regulation of DA release is modulated principally by D3 autoreceptors, whereas the regulation of DA biosynthesis and metabolism is mediated by D2 autoreceptors (Raevskii, 1998).

2. *The neuroanatomy of the basal ganglia*

The DAergic system of the brain is historically classified into the neuroanatomical groups A8-A17, with numbers increasing caudo-rostrally in the brain, starting in the midbrain with A8 at the retrorubral field and terminating with A17 in the retina. The major groups of DAergic neurons, and the most relevant to the present study, are groups A9 and A10 (SNr and VTA respectively). These groups of cells are intimately related to another large complex of brain regions called the BG. In fact, the substantia nigra (SN) is considered an integral part of this complex.
Historically the term BG was applied to the entire basal forebrain region, then only the regions primarily related to motor control were considered (caudate-putamen and GP). Then the ventral borders of the BG were expanded to include the NAcc and OT (ventral striatum) based on similarities of projections and cellular organization. Later, the subthalamic nucleus (STN) and the SN were also included. The VTA is intimately related to the SN both in cytoarchitecture and function, and therefore these two structures will be discussed together.

2.1. SN-VTA complex.

The SN receives its name from the black pigment melanin, a product of DA degradation. "Substantia nigra" literally means "black substance". This region attracted particular attention due to the fact that DAergic neurons degenerate in Parkinson's disease. Moreover, the projections of the striatonigral pathway are lost in Huntington's chorea. SN together with VTA are implicated in thought and in affective disorders such as schizophrenia and manic-depressive illness. In addition, deregulation of the DAergic system has been linked to Tourette's syndrome, attention deficit hyperactive disorder and the generation of pituitary tumors (Vallone et al., 2000). Finally, the entire complex plays a key role in the generation of reward, motivation and drug addiction. The SN is also fascinating in its general neurochemical profile. The processing occurring there resides more on the chemical specificity of the neurotransmitters than on a very precise anatomical specificity (Greenfield, 2000).

2.1.1. General topography and organization of SN-VTA

The SN-VTA lies in the ventral tegmentum of the mesencephalon, 2.5 mm long rostro-caudally and 3 mm wide medio-laterally. SN and VTA share neurochemical and functional properties, and thus form a complex structure. However, important differences exist between the two regions.
The SN comprises three parts, the SNc, a sheet of mainly DAergic (90%) neurons overlying the SNr, and the pars lateralis (SNl), a lateral extension of the SNc. The SNr is a pallidal structure composed of GABAergic neurons, which form a major output of the BG. The SNc is composed of DAergic neurons that extend posteriorly in the retrorubral field.
Medial and dorsomedial to the SNc and SNr lies the VTA. The VTA comprises the rostral VTA, the parabrachial pigmented nucleus, the paranigral nucleus, the rostral linear nucleus, the interfascicular nucleus and the caudal linear nucleus. The VTA is a more homogenous structure than the SN. 80% of the VTA cells are DAergic neurons of a wide range of sizes and shapes (Halliday and Tork, 1986; Phillipson, 1979; Phillipson, 1979; Swanson, 1982). The rat VTA is more similar to the human VTA in its cellular distribution than to the VTAs of nonhuman primates (Halliday and Tork, 1986) and is, therefore, a good model for the study of the general mechanisms of higher brain function.

2.1.2. Cytoarchitecture of SN-VTA

The SNc contains densely packed, medium-sized, DAergic neurons, approximately 11x20 µm in somal size. The SNc is divided into a ventral and a dorsal tier. Adjacent DAergic neurons in the ventral tier of SNc often give rise to closely apposed clusters of "apical" dendrites that invade the SNr (Figure 2A,D) (Bjorklund and Lindvall, 1975). These dendrites release DA (Cheramy et al., 1981) which interacts with DA autoreceptors, DA receptors on adjacent DAergic dendrites, striatonigral neurons (expressing D2 receptors), or a combination thereof. This feature defies the textbook view of how neurons work because, in contrast to the classical situation, these dendrites are found in both pre- and post-synaptic positions simultaneously.
The dorsal tier of the SNc does not contain cells with inverted "apical" dendrites; rather its neurons are typically medium-sized and fusiform (Fallon and Moore, 1978). This arrangement of separate dendritic fields is the basis for the functional separation of input and output domains in the SNc. The dorsal-most sheet of SNc neurons is continuous with the more medially placed parabrachial pigmented nucleus neurons of the VTA. Neurons of the dorsal SNc are functionally and anatomically related to VTA neurons. Instead of the usual SNc-VTA opposition, it may be best to distinguish between the ventral tier of the SNc and VTA versus the dorsal tier of the SNc (calbindin-

positive in rat), especially with regards to their cortical inputs (Figure 2B,C).

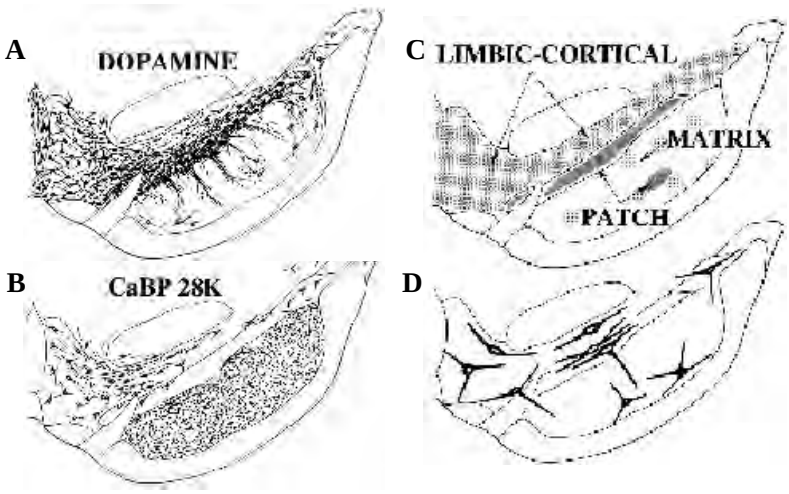


Figure 2. Coronal sections of the right side of the SN-VTA through a midrostral level. The locations of cell bodies, containing the DA (A) and 28-kDa calcium-binding protein calbindin (CaBP 28K, B), are shown. Cell types (D) and zones (C) in the SN-VTA, defined on the basis of inputs from limbic and cortical structures, and the striatum (patch and matrix). The projections from the patch overlie DAergic cell bodies in the SNc and SNr (adapted from (Paxinos, 1995))

2.1.3. Development of SN-VTA

The SN-VTA is derived from a primordial cell group in the ventral mesencephalon, which is first detectable at approximately E12.5 (Specht et al., 1981). By E17, this cell group divides to form the A8, A9, and A10 groups. DAergic fibers can be visualized by E14.5. In early postnatal development, the innervation of the caudate-putamen has an island appearance that seems to correspond to the patch-matrix organization (van der Kooy, 1984). This and the very early development of this system led to the hypothesis that SN-VTA cells might control striatal development.

2.1.4. SN-VTA connections

2.1.4.1. Inputs

The inputs to the SN-VTA are summarized in Figure 3. Striatal matrix neurons, expressing GABA, dynorphin (Dyn) and substance P (SP), project to GABAergic SNr neurons (and distal dendrites of DAergic neurons). GABA receptors are numerous in SNr, but predominantly GABA-A are present (Bowery et al., 1987). The striosomes or striatal patch neurons, containing GABA and enkephalin (Enk), send their axons to ventral SNc neurons (with dendrites in SNr). Opioid peptides may facilitate neurotransmission in SN-VTA DA neurons through mu-receptor-mediated inhibition of inhibitory interneurons in the SN-VTA (Johnson and North, 1992). Also κ and δ opioid receptors are present in SN-VTA (Clarke and Pert, 1985).

The STN is the source of excitatory glutamatergic (Glu-ergic) projections to SNr neurons (Groenewegen and Berendse, 1990). The GP and the ventral pallidum (GABAergic) project to SN and VTA (Haber and Nauta, 1983). These projections are organized topographically (ventrodorsally) (for review: (Gerfen, 1992; Gerfen, 1992)). Other inputs include the amygdala (Shinonaga et al., 1992), the hypothalamus (Nauta et al., 1978), the preoptic area, and the cortex, especially the frontal and the cingulate areas (Wyss and Sripanidkulchai, 1984), all sending their axons to both the SNc and the VTA (especially the dorsal-most tier). Prefrontal cortex projections use glutamate (Glu) in the SN and aspartate in the VTA. The AMPA-receptors are predominant in the SNc (Petralia and Wenthold, 1992), and NMDA-receptors in the SNr (Monaghan and Cotman, 1985).

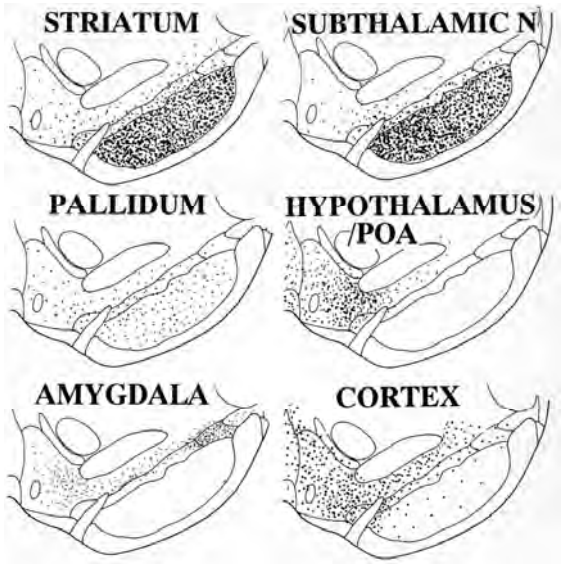


Figure 3. Inputs to the SN-VTA from the brain areas noted (adapted from (Paxinos, 1995)).

The inputs from the brainstem include serotonergic projections from the dorsal and median raphe nuclei (primarily to SNr) (Steinbusch, 1981). Serotonin (5-HT) receptors are largely confined to striato-nigral terminals of the SNr (Palacios et al., 1990; Pazos et al., 1987). The noradrenergic inputs are represented by the projections en route from the locus coeruleus to the forebrain through the VTA (Fallon and Moore, 1978) as well as cholinergic inputs from the

pedunculopontine and tegmental nuclei. The SN-VTA themselves contain some cholinergic bodies (Gould et al., 1989; Martinez-Murillo et al., 1989; Martinez-Murillo et al., 1989).

2.1.4.2. Outputs

2.1.4.2.1 *DAergic*

The DAergic projections from the SN-VTA are summarized in Figure 4. Ventral and intermediate sheets of the SNc and the ventro-lateral VTA project to the caudate putamen (nigrostriatal pathway), whereas the dorsal and middorsal VTA and medial SNc send their axons to the NAcc and OT in the ventral striatum (mesolimbic pathway). Other targets include the amygdala (from the VTA, the lateral SNc and the SNl) and the lateral septum (from the paranigral nucleus and the medial SNc). The dorsal-most sheet of the SNc and the VTA send their projections to the prefrontal, cingulate, perirhinal, and entorhinal cortices (mesocortical pathway). In addition there are sparse DAergic projections to other regions: the cerebellum, the dorsal lateral geniculate nucleus, the hypothalamus, the raphe, the hippocampus, the ventral pallidum, and the locus coeruleus. Cortical, striatal and pallidal projections arise topographically and predominantly (95%) ipsilaterally. Many DAergic projections also contain cholecystokinin (CCK) and other neuropeptides. Although cortical outputs come from the dorsal tier of the SNc and the VTA as one complex, the outputs to the NAcc remain primarily reserved to the VTA.

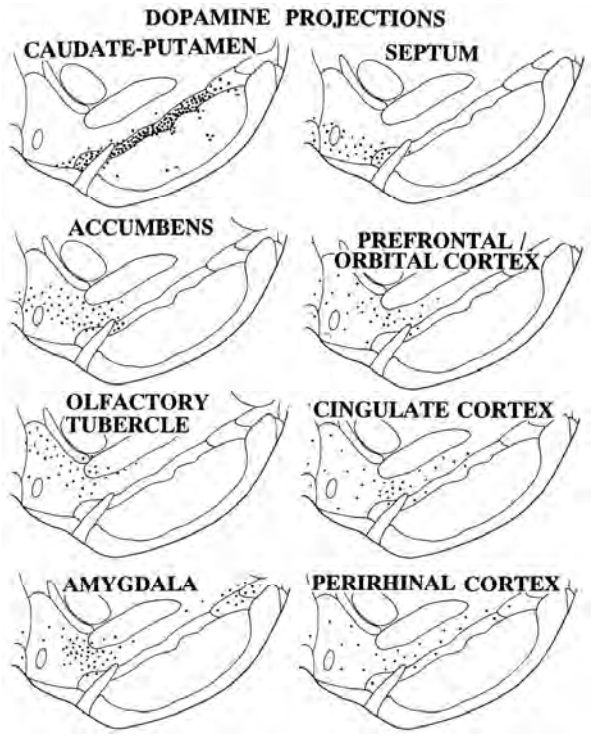


Figure 4. Localization of DAergic neurons in the SN-VTA, giving rise to mesotelencephalic projections (adapted from (Paxinos, 1995)).

2.1.4.2.2 *Non-DAergic*

The SNr projects to the thalamus (ventromedial, dorsomedial and intralaminar nuclei, in turn projecting essentially to the frontal cortex), the superior colliculus (Williams and Faull, 1985) and mesencephalic motor regions (pedunculopontine nuclei and surrounding mesopontine tegmentum) (Faull and Mehler, 1978). SNl neurons send their axons to the inferior colliculus (Moriizumi et al., 1992), whereas the VTA projects to the visual cortex (Dinopoulos and Parnavelas, 1991). In addition, there are some other, less dense, projections.

2.1.5. General principles of SN-VTA inputs and outputs organization

SNr neurons receive an excitatory input from the STN, which is under the direct influence of the cortex and the striatum. The GABAergic neurons of SNr, but also the "apical" dendrites of DAergic neurons of the ventral tier of the SNc will be influenced by GABA, SP and Dyn-containing terminals arising from the matrix of the striatum. This allows striatal inputs to influence both systems (GABAergic and DAergic) at the same time. DAergic neurons of the ventral tier receive motor inputs, whereas the dorsal tier, which receives less inputs from the striatum, is more under the influence of cortical, hypothalamic, amygdaloid and other "limbic" regions of the forebrain. The differential inputs are mirrored by differential ion conductances in these various sectors.
Together, SN-VTA neurons can integrate both limbic and motor activity. A distinction between limbic and nonlimbic extrapyramidal zones in the SN-VTA is based on the dorsal-ventral dichotomies of inputs, outputs, and dendritic domains of the DAergic neurons. Connections within the DAergic system are summarized below and in Figure 5:

1. frontal and cingulate cortices, as well as NAcc and OT, project to the dorsal tier (direct limbic) of the SN-VTA
2. limbic cortices project to the striosomes that, in turn, innervate the intermediate neurons between the dorsal and ventral tier (indirect limbic)
3. sensorimotor cortices project to the striatal matrix whose neurons, in turn, send their axons to the SNr (with dendrites of the ventral tier) (motor)

At the level of outputs, the dorsal tier projects to the cortex, the ventral striatum and limbic structures as well as to the striatal matrix, whereas the ventral tier projects to the dorsal striatum (striosomes). Overall, DA facilitates most of the loops and outputs in the extrapyramidal motor system.

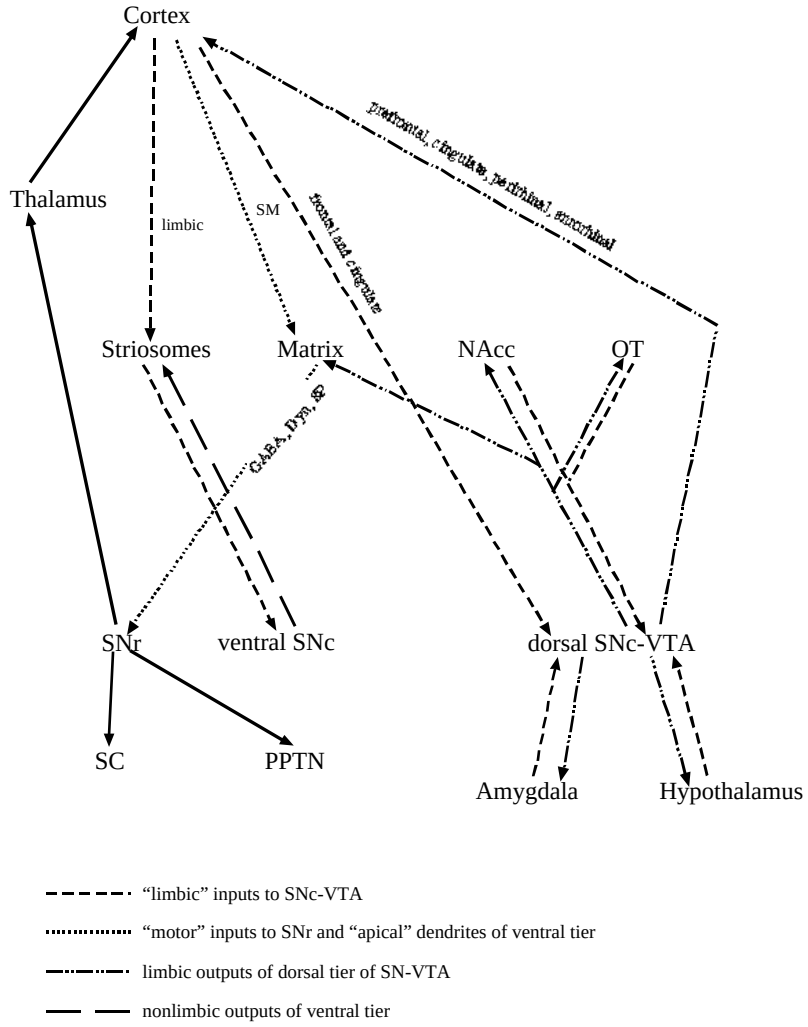


Figure 5. General scheme of DAergic system connections. NAcc: nucleus accumbens, OT: olfactory tubercle, PPTN: pedunculopontine tegmental nucleus, SC: superior colliculus, SM: sensorimotor cortical projections, SNc: substantia nigra, pars compacta, SNr: substantia nigra, pars reticulata, VTA: ventral tegmental area.

2.2. Basal ganglia

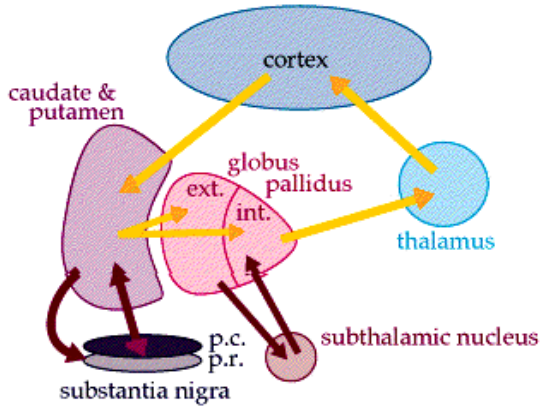


Figure 6. General scheme of BG components and their interconnections in primates. The system is slightly different in rat. The external segment of primates is represented in rat by GP (lateral GP in Rat Brain Atlas (Paxinos and Watson, 1998)). Internal pallidal segment of primates is represented in rat by entopeduncular nucleus (medial GP in Rat Brain Atlas (Paxinos and Watson, 1998)).

2.2.1. BG topography, neurochemistry and cytoarchitecture

2.2.1.1. Striatum

The striatum has different levels of compartmentalization. A functional separation into dorsal (dorsolateral) and ventral (ventromedial) striatum matches the type of information processed and the particular topography of their efferents. The dorsal striatum receives the inputs mostly from the neocortex while the ventral striatum is innervated by the axons from the allocortex and mesocortex. The differences are gradual rather than abrupt, and significant overlap between allocortical-mesocortical-amygdaloid projection fields exists. Accordingly, a precise boundary between the ventral and the dorsal striatum is difficult to draw.

The dorsal striatum is separated into caudate and putamen, although in rats this distinction is not significant because of poor development of the internal capsule. The ventral striatum comprises ventromedial parts of the caudate-putamen, the NAcc, and the medium-sized cells in the OT (Heimer et al., 1991). The NAcc is further subdivided into the core and the

surrounding shell (Zaborszky et al., 1985). The rostral pole of the NAcc is a distinct region because it does not show a core-shell dichotomy (Heimer and Alheid, 1991; Zahm and Heimer, 1993).
BG display characteristic border regions sharing the properties of neighboring structures. For example, the shell of the NAcc has neurochemical and connectional features more common to the closely localized extended amygdala, e.g., strong CCK and angiotensin II (ATII) immunoreactivity and projections to the hypothalamus (Heimer and Alheid, 1991; Zaborszky et al., 1985). However, these two structures represent separate, even if highly interacting, functional anatomical entities (Zahm, 1998). The extended amygdala projects to the posterior lateral hypothalamus with major final outputs in autonomic centers at the brainstem, whereas the shell of the NAcc and the ventromedial ventral pallidum project throughout the lateral hypothalamic area and send essentially all BG projections to the prefrontal cortex.
A second level of compartmentalization of the striatum is its "striosomal", or patch-matrix organization (Graybiel et al., 1978). A relatively constant ratio of 15% striosomes to 85% matrix is maintained in the striatum of rat, rhesus monkey and human. The striosomes form a branched three-dimensional labyrinth within the matrix (Groves et al., 1988). The average number of striosomes stays constant from rat to human with a corresponding increase in striosome volume. The histochemical markers used for the matrix compartment are AChE or calbindin (in the rat), whereas the mu-opiate receptor binding and immunohistochemical staining for enkephalin are the markers of striosomes (Johnston et al., 1990; Pert et al., 1976). Striosomes and the matrix as defined in the dorsal striatum are not present in the ventral striatum.
In the rat 90-95% of striatal neurons are medium spiny neurons. These cells have perikaryi with diameters ranging between 12 and 18 µm and 4-8 dendrites that are relatively devoid of spines proximal to where they first branch, but beyond are very densely spined all the way to their tips, which are typically 100-250 µm away from the cell body. GABAergic medium spiny neurons are both projection neurons and interneurons. The interneurons with their dendritic tree form the basket on the cell bodies of projection neurons. These interneurons receive major inputs not only from the cortex, but also from downstream BG nuclei, and thus present an important level of regulation for projection neurons. Each projection neuron receives inputs from about 50 GABAergic interneurons.
A minor but critical proportion of the striatal neurons (less than 2% in rats) are cholinergic interneurons (Phelps et al., 1985; Walaas and Fonnum, 1979) which play an important role in the integration of signals and in the modulation of the striatal output (Dimova et al., 1993; Pickel and Chan, 1991).

2.2.1.2. Pallidum

The pallidum consists of a larger external component, the GP, and a smaller intracapsular component, the entopeduncular nucleus (EP), which also includes the SNr. In rodents the EP represents the homologue of the primate internal pallidal segment. The pallidum is usually separated into the ventral and dorsal divisions but, as in the striatum, there is no way to mark a border in-between. Because of inputs from the ventral striatum, the ventral pallidum is positive for SP (Haber et al., 1985; Haber and Nauta, 1983; Haber and Watson, 1985) and represents both external and internal pallidal segments (Alheid and Heimer, 1988; Groenewegen et al., 1993). The SNr shares many histological features with the internal segment of the GP (i.e. the EP). Both are characterized by dense SP (Inagaki and Parent, 1984), epidermal growth factor (Alheid and Heimer, 1988; Fallon et al., 1984) and GABA immunoreactivity (Oertel et al., 1982).

2.2.1.3. Subthalamic nucleus

The STN is an ovoid nucleus partly embedded in the dorsomedial part of the pes pedunculi. It contains a homogeneous collection of densely packed, medium-sized Glu-ergic neurons with relatively long dendrites, which contain few or a moderate number of spines (Yelnik and Percheron, 1979). In the rat, as opposed to primates, the distal dendrites occasionally cross the borders of the nucleus (Hammond and Yelnik, 1983). The STN, like the striatum, influences motor activities primarily through its prominent projections to the EP and the SNr. Accordingly the majority of STN neurons are projection neurons, whose axons bifurcate projecting to both output nuclei and in addition emit few collaterals that are distributed within the nucleus.

2.2.2. BG connections

A detailed scheme of BG subregions in the rat brain is presented in the Figure 7.

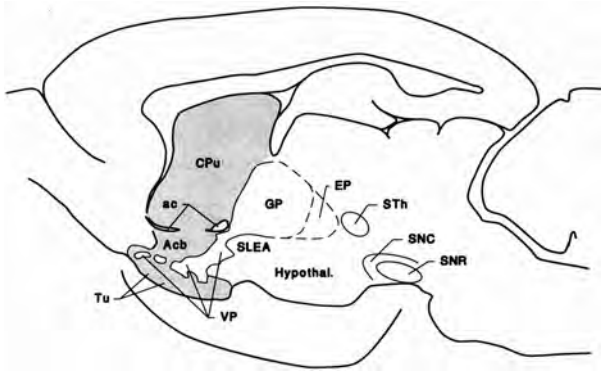


Figure 7. BG Outline. The BG and a few other structures (hypothalamus and sublenticular extended amygdala) are identified in a parasaggital section of a rat brain. ac, anterior commissure; Acb: nucleus accumbens; CPu, caudate-putamen; EP, entopeduncular nucleus; GP, globus pallidus; SLEA, sublenticular extended amygdala; SNC, substantia nigra, pars compacta; SNR, substantia nigra, pars reticulata; STh, subthalamic nucleus; Tu, olfactory tubercle; VP, ventral pallidum.

2.2.2.1. Inputs

All cortical areas are connected to the striatopallidal system, and the heterogeneity of the system itself probably serves different needs of various cortical regions (Figure 8).

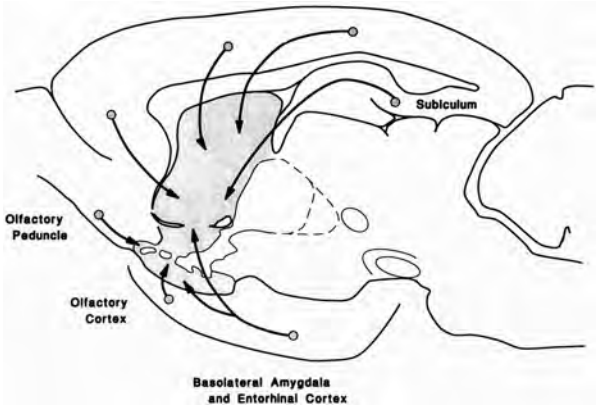


Figure 8. Schematic drawing of the corticostriatal projections.

The distinction between dorsal and ventral striatum is based on the difference at the level of cortical inputs. Cortical areas themselves receive the input from different thalamic nuclei as follows:

Ventroanterior-ventrolateral nuclei of the thalamus project to the motor-premotor cortex with the final output in the dorsal part of the striatopallidal system, while the mediodorsal thalamic nucleus projects to prefrontal cortex neurons sending, in turn, their axons to the ventral striatopallidal system.

Corticostriatal projections use Glu (Christie et al., 1987; Fonnum et al., 1981). CCK may also be involved (Meyer et al., 1982; Zaborszky et al., 1985). Cortical projections reach the striatum in a direct fashion, resulting in a topographic projection system, with all parts of the cortex projecting to the adjacent parts of the caudate-putamen (Figure 8). The projections to the ventral striatum from both the allocortex and the medial mesocortex (retrosplenial, anterior cingulate, prelimbic, and infralimbic areas) and from the lateral mesocortex (sulcal areas, agranular insular area, and perirhinal area) project topographically to a ventral striatal territory, which includes the OT, the NAcc, and a large ventromedial territory of the caudate-putamen. The involvement of the ventromedial caudate-putamen is more extensive for the mesocortical areas than for the allocortex. Each allocortical and mesocortical area projects bilaterally, with an ipsilateral predominance and with longitudinally extended striatal projection fields (Beckstead et al., 1979).

Amygdalostriatal projections originate mainly in the cortical-like basolateral amygdaloid complex (Figure 8) (Carlsen and Heimer, 1988).

The thalamocortical projections are a major source of topographically organized striatal afferents that originate primarily in the midline and intralaminar nuclei of the thalamus (Beckstead, 1984; van der Kooy, 1979). These thalamostriatal projections also include afferents from areas such as the ventral thalamic nuclei (Jones and Leavitt, 1974), the lateral posterior and posterior thalamic nuclei (Veening et al., 1980). The thalamocortical projections are topographically organized in anteroposterior and mediolateral directions. The fact that the striatopetal thalamic nuclei also project to the cortex suggests that these two projections may, at least to some extent, represent collaterals of single neurons (Jones and Leavitt, 1974). The thalamostriatal input is excitatory and presumably Glu-ergic (Kitai et al., 1976), but afferents from the thalamus and from the cerebral cortex do not converge on the same striatal spiny neuron (Dube et al., 1988).

Mesostriatal DA projections represent about 20% of striatal inputs (Figure 9A). DAergic terminals synapse on the very same neurons as corticostriatal terminals, NMDA-receptors being expressed on the spinal head and DA receptors on the spinal neck. Other ascending afferent projections include 5-HT from the dorsal raphe to striatum and pallidal complex (Figure 9A) (Azmitia and Segal, 1978; Veening et al., 1980) and seemingly cholinergic projections (Woolf and Butcher, 1986) from the mesopontine reticular formation (including the pedunculopontine tegmental nucleus) (Figure 9B).

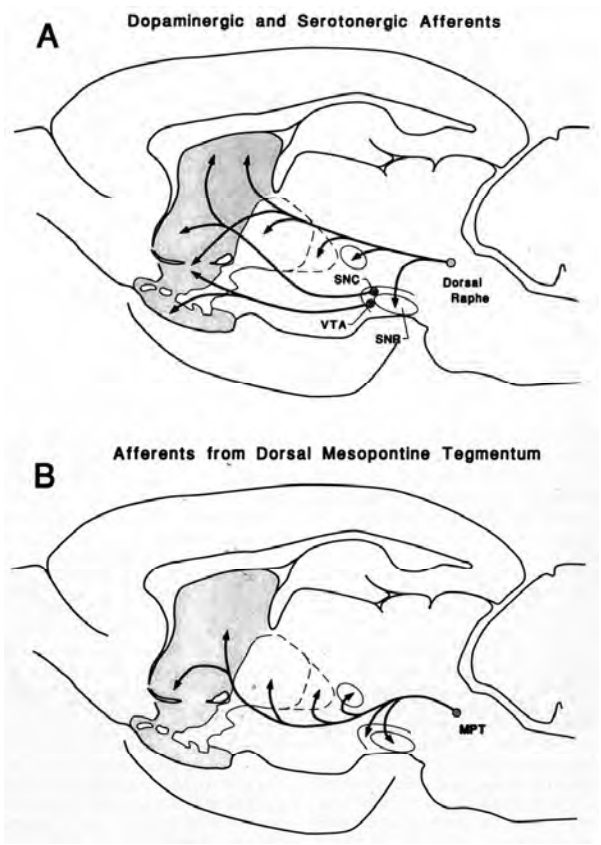


Figure 9. Mesencephalic inputs to the BG. MPT, dorsal mesopontine tegmentum; VTA, ventral tegmental area. See Figure 7 for other abbreviations.

2.2.2.2. Outputs

The striatum projects either directly or indirectly via the STN to BG output nuclei (GP, EP, and SNr) (Figure 10). These three output regions share many similarities, but also differences. For ex., only the ventral pallidum, EP and SNr receive a dense SP/Dyn

innervation from the striosomes (Beckstead and Kersey, 1985; Haber and Nauta, 1983; Vincent et al., 1982). Alternatively, the EP and SNr do not receive a comparable Enk-ergic input from the striatal matrix in the rat. Only the GP and the ventral pallidum project to the STN.

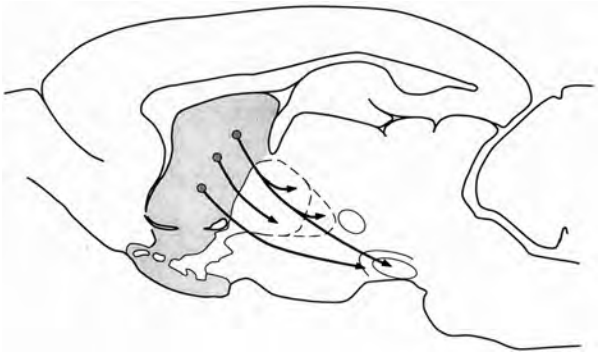


Figure 10. Schematic drawing of dorsal striatofugal projections.

Spiny projection neurons in the dorsal striatum can be differentiated onto several groups:

1) Neurons with local axon collaterals restricted to the parent dendritic domain (most common), including Enk-ergic striatopallidal projections (expressing D2 receptors) and SP/Dyn-ergic striatonigral projections (expressing D1 receptors (Kawaguchi et al., 1990)), which may send a collateral axonal branch to the EP;
2) Neurons with more widely spread collateral branches (Bishop et al., 1982; Kawaguchi et al., 1990).

The matrix compartment of the striatum projects to the SNr, which contains GABA-ergic neurons and the distal dendrites of DA neurons (Gerfen et al., 1985; Gerfen and Sawchenko, 1985), whereas the projections to the ventral tier of the SNc originate in striosomes and synapse at a neurochemically distinct subset of DAergic cell bodies. This represents two parallel corticostriato-pallidonigral pathways through the patch-matrix compartments, which differentially regulate the BG output directly or through a loop on DAergic cells (Gerfen, 1992; Gerfen, 1992). The ventral striatum projects primarily to DA neurons which innervate the matrix. The core of the NAcc projects to the dorsolateral part of the ventral pallidum, EP, the lateral part of the VTA, and to the SNc, which affects the activity of the dorsal striatum via the DA system (Wouterlood et al., 1992). In contrast, the shell of the NAcc and the rostral pole project to the ventromedial parts of the ventral pallidum, EP, the VTA, and the mediodorsal SNc (which are the typical projections for BG structures), and also to the extended amygdala and the lateral preoptic-hypothalamic continuum (Zahm and Heimer, 1993), thus resembling the extended amygdala projections. The shell also has more pronounced projections to the mesopontine reticular formation and the periaqueductal gray.

The BG output nuclei project to the cortex with a relay in the thalamus as well as the superior colliculus, the lateral habenula, and the reticular formation in the general region of the pedunculopontine tegmental nucleus.

In more detail, the GP projects to STN, EP, SNc and SNr, the superior colliculus (Carter and Fibiger, 1978) and carries projections back to the striatum and to the cortex (Figure 11A) (Spooren et al., 1996; Spooren et al., 1991). The ventral pallidum, in turn, projects to STN and SN (from dorsolateral part), the mediodorsal thalamus, EP-lateral hypothalamic area, and VTA (from ventromedial part).

EP and SNr project to the thalamus (ventromedial-ventrolateral complex, the centromedian-parafascicular complex, the mediodorsal nucleus and the adjacent intralaminar nuclei), the superior colliculus and the pedunculopontine tegmental nucleus (Figure 11B) (Jackson and Crossman, 1981).

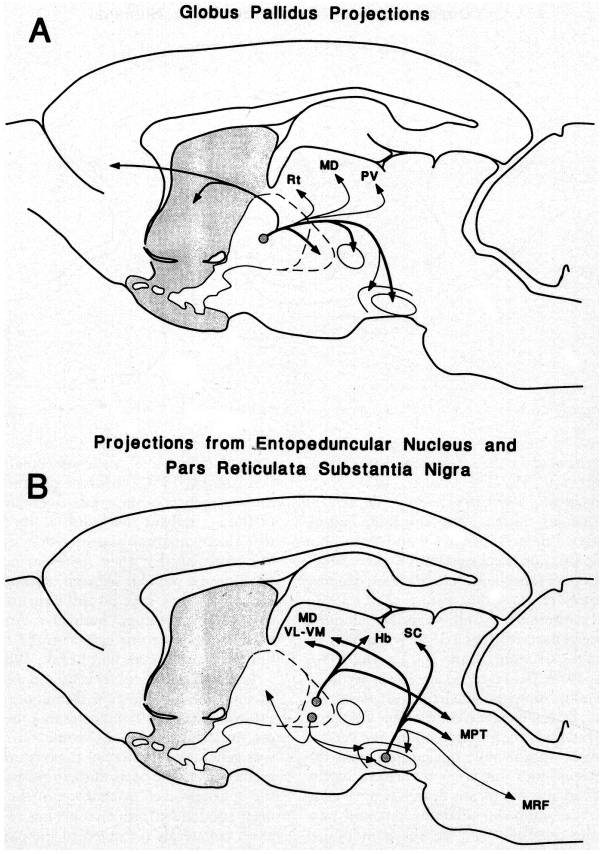


Figure 11. Major output BG projections. Hb, habenula; MD, mediodorsal thalamic nucleus; MPT, dorsal mesopontine tegmentum; MRF, medullary reticular formation; PV, paraventricular thalamic nucleus; Rt, reticular thalamic nucleus; SC, superior colliculus; VL-VM, ventrolateral-ventromedial thalamic complex.

Thus, both the lateral and the ventral pallidum in their output projections can undergo two alternative ways, which basically defines the ***direct*** and the ***indirect*** processing pathways in BG, the latter using the STN as a relay station. Accordingly, the two major projections of the STN are the GP and the SNr (Figure 12) (Kita et al., 1983). An overwhelming majority of STN neurons send a descending axonal branch to the SN and an

ascending branch that terminates in both the EP and the GP (Parent and Hazrati, 1995; Sato et al., 2000).

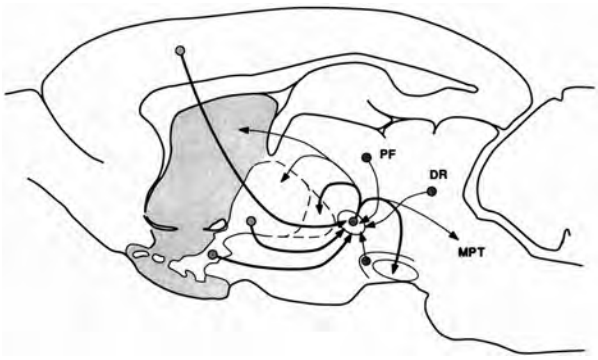


Figure 12. STN connections. DR, dorsal raphe nucleus; MPT, dorsal mesopontine tegmentum; PF, parafascicular thalamic nucleus.

2.2.3. Information processing in BG

2.2.3.1. Parallel vs convergent BG circuitry

The question of whether cortical inputs are processed in a parallel or a convergent way in the BG is still debated. Different facts argue for both possibilities. For example, pallidal neurons have perpendicular dendritic trees allowing for cortical inputs to converge on several neurons simultaneously. But the same cortical axons also make multiple synapses en passant on the same neuron, thus forcing this neuron to be its preferential target. This latter observation supports the idea of preferentially parallel processing.

BG outputs represent a significant condensation of the information presented to the BG by corticostriatal afferents. From these outputs a considerable proportion is directed to subcortical and brain stem targets, but also back to the cortex as reentrant circuits along basal ganglia-thalamocortical pathways. The question about the type of processing remains open for the entrant-reentrant pathways. One hypothesis proposes the parallel processing, i.e. the BG-processed information returns back to the same cortical area where the corticostriatal afferents originated. This hypothesis is well supported in regard to "motor" and "oculomotor" circuits, originating in supplementary motor area and frontal eye field, respectively (Alexander et al., 1990; Alexander et al., 1986). The same hypothesis was postulated for BG-thalamocortical circuit, originating from the prefrontal cortex (Groenewegen et al., 1993; Groenewegen et al., 1999). An alternative viewpoint is that the output of ventral BG-thalamocortical pathways is directed largely at cortical areas adjacent to those in which the circuits originate (Brog et al., 1993; Zahm and Brog, 1992), thus forming the spiral BG-thalamocortical reentrant circuits. Such a spiral pathway appears to proceed, for example, from the piriform cortex and other allocortical areas toward premotor/supplementary motor areas through the ventral BG (Brog et al., 1993; Zahm and Brog, 1992). The different spiral tours, passing through the ventral BG, form the distinct channels with pronounced morphological and neurochemical specialization, particularly in regard to DAergic neurotransmission (Deutch and Cameron, 1992; Zahm, 1991). This configuration allows for differential modulation by DA of cortical impulses conducted through various channels of the ventral BG. Such differential DA modulation exists already in the dorsal striatum and is the basis of direct vs indirect information processing.

2.2.3.2. Direct vs indirect pathway

The output projections of the BG are organized in two parallel pathways (Figure 13). The dorsal striatum contains two types of medium spiny neurons, different in their DA receptor expression and output projections. D1 containing GABA-ergic neurons, also synthesizing SP and Dyn, project directly to the internal segment of the GP (EP in rats) and SNc, thus forming the direct pathway. D2-expressing GABA-ergic neurons, also containing Enk, project first to the external segment of GP and constitute the first part of the indirect pathway. The axons of the external segment project to the STN which, in turn, sends its axons to both SNc and SNr as well as back to the external segment.

SNc, making DAergic projections to both types of striatal neurons, will however regulate them in opposite ways; inhibitory through D2 receptors on the indirect pathway and excitatory through D1 receptors on the direct pathway (Gerfen et al., 1990). In addition, the neurons of the direct pathway of the striatum receive more pronounced inputs from the thalamus than ones of the indirect pathway. By counteracting the effects exerted by DA, the opioids expressed by GABAergic striatal neurons, serve as negative feedback elements to prevent the excessive stimulation of striatal neurons in which they are expressed (Steiner and Gerfen, 1999; Steiner and Gerfen, 1998). DA neurons are, in turn, regulated by striatal inputs, originating exclusively in striosomes and thus carrying information from the limbic cortex (Gerfen et al., 1985; Jimenez-Castellanos and Graybiel, 1989).

Similar parallel loops of direct and indirect pathways cross the ventral striatum and pallidum and include the prefrontal cortex, a relay in mediodorsal thalamic nucleus and the DAergic input from the VTA. Even if the ventral pallidum is not morphologically separated into the external and internal segments, the two pathways remain clearly separated.

The clinical importance of such parallel processing of information is clearly demonstrated in Parkinson's disease (PD). In PD patients the DAergic pathway between the striatum and SN is degenerated and DA levels in the striatum are drastically reduced. The depletion of DA disbalances the direct and indirect pathways from the striatum, which causes the thalamus to be overstimulated. As a result, the frontal cortex is less activated accounting for most of the Parkinsonian symptoms.

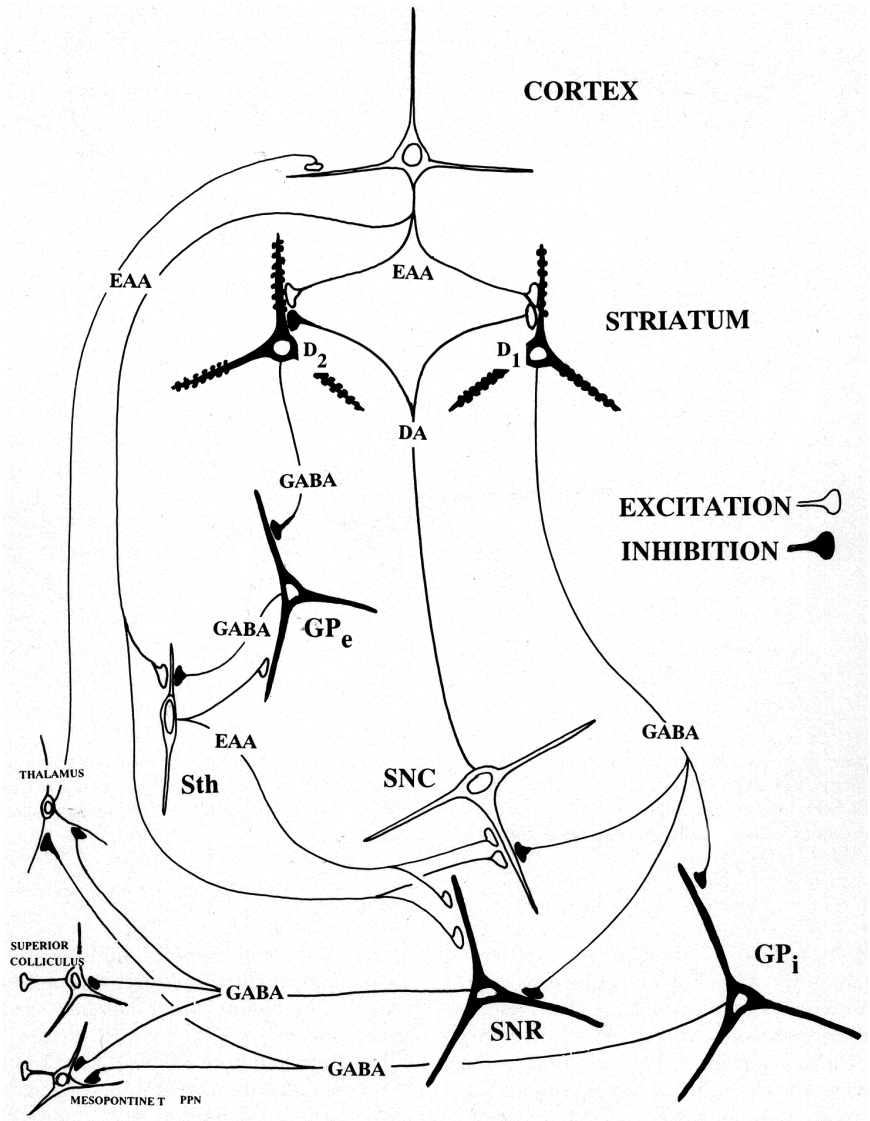


Figure 13. Major input-output relationships of the SNc (DAergic) and SNr (GABA-ergic) neurons with other components of the extrapyramidal motor system, illustrating the separation of "direct" and "indirect" pathways through the BG. Neurons in black are inhibitory GABA-ergic neurons, and neurons in white are excitatory neurons. The SNc DAergic neurons (in white) are both excitatory and inhibitory (on D1- or D2-expressing medium spiny neurons in the striatum, respectively). DA, dopamine; EAA, excitatory amino acids; GPe, external segment of the globus pallidus; GPi, internal segment of the globus pallidus; PPN, pedunculopontine tegmental nucleus; SNC, substantia nigra, pars compacta; SNR, substantia nigra, pars reticulata; Sth, subthalamic nucleus.

2.2.3.3. Reward and motivation and an application to drug addiction

Reward serves as the basis for reinforcement learning. The psychological concept of positive reinforcement was formulated by Thorndike (1911) as follows: "Any act which in a given situation produces satisfaction becomes associated with that situation so that when the situation recurs the act is more likely than before to recur also". Thus, the neuroanatomical basis of reinforcement should include two systems: one executing the motor act, and the second, able to detect the positive effects produced by this act and consequently specifically facilitate the occurring of this act in the same situation in the future. At the cellular level the reinforcement is explained by the phenomenon of heterosynaptic plasticity: the synaptic strength of one pathway (leading to motor activity in this case) is modified by the activity in another pathway (reward-detecting) (Wickens and Kötter, 1995). It has been known for a long time that the corticostriatal inputs constitute the motor pathway through the BG. Concerning the second pathway, multiple studies suggest the role of DAergic neurons in the activation of behavioral reinforcement. The evidence comes from studies on electrical brain stimulation, lesions and psychopharmacology. For example, electrical stimulation of the medial forebrain bundle, the main pathway by which the DAergic terminals reach the cortex, is highly reinforcing. The most effective electrical brain stimulation reward sites are the ones that directly or indirectly activate the DAergic neurons in the midbrain (Wise and Hoffman, 1992). These and other data suggest, but do not prove, that DAergic neurons projecting to the neostriatum are a major branch of a "final common pathway" for positive reinforcement (Wise and Bozarth, 1984). In this context, positive reinforcers stand for signals that are able to reinforce the behavior leading to their appearance.

To play a role in reinforcement, DAergic stimulation should be able to strengthen the corticostriatal pathway. In fact, it was shown that application of DA can produce long-lasting facilitation of synaptic transmission in non-DAergic pathways. Concerning the

striatal output, DA increases output from the most active cells (a small number of neurons), and decreases output from the less active cells, thus augmenting the "signal-to-noise" ratio. The increased activity is probably due to the strengthened excitatory input from the cortex. Actually, the immediate effect of DA release is probably a reduction in postsynaptic excitability, thus permitting only the strongest inputs to bypass the threshold. In the long term, however, these focused rare projections will be potentiated (Schultz et al., 1995). At the behavioral level, the increased behavioral activity occurs in limited categories of response.

The accepted function of DAergic neurons as reinforcers of corticostriatal input does not explain the precise nature of DAergic activation itself. An early theory explained the role of the DAergic system as one that determines the relative pleasure of incoming stimuli (Wise, 1982). This theory suggests that the DA system mediates the pleasure produced by natural reinforcers. Upon addition of DA antagonists, all pleasures lose the ability to arouse the animal, therefore the term "anhedonia hypothesis" was given to this theory, which was later rejected by Wise himself (Wise, 1994). Another theory suggests the role of the DA system in attributing the incentive salience to otherwise neutral rewards (Berridge and Robinson, 1998). This theory makes a distinction between the phenomena of "liking" and "wanting", and their brain substrates, in relation to a particular stimulus. The DAergic system is related to "wanting", whereas the brain substrate of "liking", i.e. the brain structure that decides if a particular stimulus is pleasant, is not known so far. Such a structure, which would be the origin of the primary reinforcement, could be, according to different authors, the amygdala, the lateral hypothalamus (Hoebel et al., 1989; Wise and Bozarth, 1984), the pedunculopontine tegmental nucleus or the subthalamic nucleus. One example, illustrating such a distinction between "wanting" and "liking" in relation to DA, is the opioid action. Both mu and kappa receptor agonists are addictive; however, the self-administration of kappa-agonist is rapidly extinguished when a progressive ratio schedule compared to heroin. Kappa agonist administration, inversely to heroin, decreases the DA concentration in the NAcc. This suggests that changes in accumbal DA do not correlate with the ability of the stimulus to induce reward or aversion, but with the drive to reach a positive reinforcer (Marinelli et al., 1998).

A reward has two functions in the process of conditioning. First it brings about learning of a new behavioral response, and second it has the capacity of maintaining an established behavioral response. The DA neurons are activated only in response to unpredicted rewards, i.e. primary reinforcement as well as secondary reinforcement signals, suggesting their role in the first function of reward, related to learning. In contrast, striatal, amygdalar and cortical responses to reward occur independently on the level of prediction, suggesting a function in the maintaining of conditioning. In this case the activation of striatal neurons will be accomplished probably by the input from the amygdala (Schultz et al., 1995). The two theories do not explain the ability of DAergic neurons to respond to unpredicted stimuli and then switch to respond to a conditioned stimulus upon learning. This particular pattern of activation can be well explained by adaptive critics-actor model.

The adaptive critic is a device that learns to anticipate reinforcing events in a way that makes it a useful coordinator of another component, the actor, that adjusts behavior in order to maximize the frequency or magnitude, or both, of reinforcing events (Barto, 1995). In this model the matrix of the striatum would be the actor, and the striasomes the critics. The first appearance of the stimulus preceding the reward will be followed by a slight activation of striosomal medium spiny neurons (small SPs response peak in Figure 15). This in turn results in a raise of intracellular Ca and involvement of CaM followed by CaM-PKII activation (Figure 14 and Figure 15). This process will be slow and retarded given the low affinity of CaM for PKII. The activity of adenylyl cyclase will also be slightly increased by a calcium-dependent mechanism. Later on, the reward (primary reinforcement) will be detected by the DAergic system, as can be measured by the increasing concentration of DA in the striatum or other output structures, and electrophysiological measurements of phasic discharge of DAergic neurons. DA will activate the adenylyl cyclase through D1 receptors. The maximum activity of adenylyl cyclase is achieved through activation of both calcium- and D1 receptor-dependent pathways (Ahlijanian and Cooper, 1988). The resulting PKA activation will raise cAMP concentration which, in turn, increases the amount of phosphorylated DARPP (Walaas et al., 1983), which is a potent inhibitor of protein phosphatase 1 (Hemmings et al., 1984). This results in DARPP-mediated disinhibition of CaM-PKII autophosphorylation which prolongs the activated state of the complex by drastically increasing the affinity of PKII for CaM. The CaM-PKII complex will then be able to potentiate Glu receptors, a process leading to LTP (dashed trace labeled LTP in Figure 15).

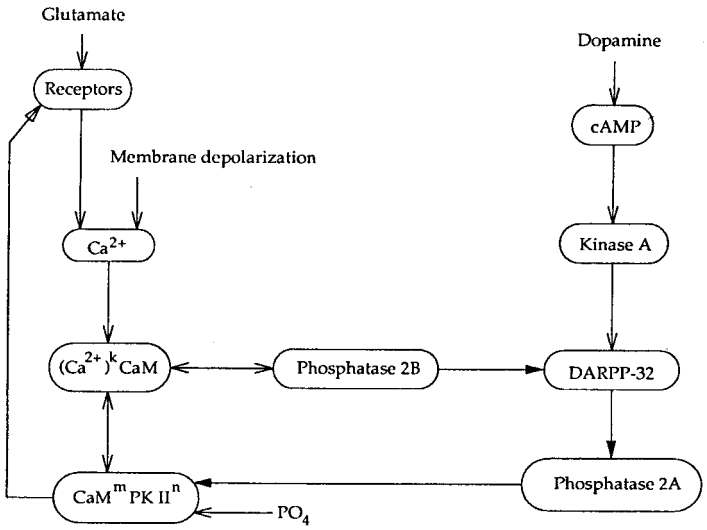


Figure 14. Cellular model of interactions for learning of earlier predictors of reinforcement. See description in text. Open arrowheads signify excitation or activation, black arrowheads signify inhibition or deactivation, and the triangular arrowhead signifies a possible potentiation or depression of receptors. Ca^{2+}, free calcium ions; $(Ca^{2+})^{k}CaM$, calcium-activated calmodulin with *k* (up to four) bound calcium ions; $CaM^{m}PK\ II^{n}$, activated -calmodulin-dependent protein kinase II with *m* pockets occupied by activated calmodulin molecules and *n* sites phosphorylated; PO_4, phosphate group; cAMP, the second messenger cyclic adenosine monophosphate; DARPP-32, DA- and cAMP-regulated phosphoprotein. (The Figures 14-17 are adapted from (Houk et al., 1995)).

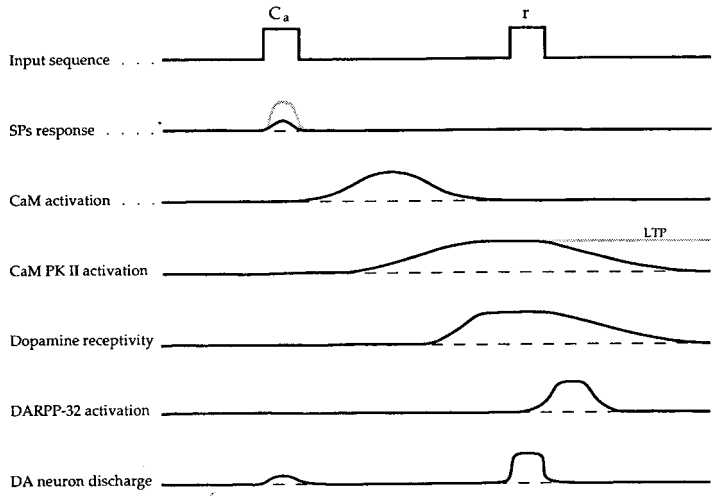


Figure 15 The interplay of intra- and extracellular events in creating long-term potentiation of a predictor of reinforcement. See text for details. Ca, reinforcement-preceding context (motor act); SPs, spiny neurons in striosomal compartments of the striatum; LTP, long-term potentiation. For other abbreviations see Figure 14.

Given the fact that the DA signal is pulsatile in nature, the synaptic modification mechanism probably has strict temporal requirements, which is consistent with behavioral observations. Moreover, DA release must occur while the calcium concentration is elevated, which means shortly after activation of a corticostriatal synapse, thus also providing the specificity of the reinforcement signal (Wickens and Kötter, 1995). The next appearance of a predicting reward stimulus (now a conditioned stimulus) will highly activate the striosomal neuron because of its potentiated state (dashed peak of SPs activation in Figure 15), which will, in turn, act on the DAergic neuron through both the direct and the indirect pathways. Given the particular kinetics of this action, known from electrophysiological studies (fast activation by indirect input, slow inhibition by direct pathway), DAergic neurons will be activated at the time of the conditioned stimulus and then inhibited during the primary reinforcer itself (Figure 16 and Figure 17). This DAergic signal, "corrected" by adaptive critics (striosome), will be transmitted to matrix neurons, reinforcing the corticostriatal inputs active first during the primary reinforcement, then during the secondary reinforcement (conditioned stimulus). This will result in the effective reinforcement produced by the stimulus.

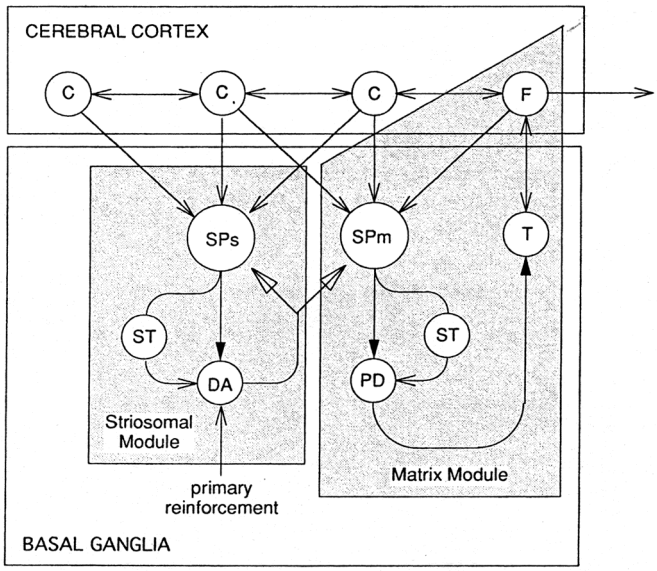


Figure 16. Modular organization of the basal ganglia, including both striosomal (critic) and matrix (actor) modules. SPs-to-DA represents the direct pathway. DA-to-SPm signal is the output of striosomal module and will account for an effective reinforcement produced by a particular stimulus. The signals in F neurons function as the ascending outputs of matrix modules. Open arrowheads signify excitation, black arrowheads signify inhibition, and triangular arrowheads signify neuromodulation. C, cerebral cortical columns; F, columns in frontal cortex; SPs, spiny neurons in the striosomal compartment of the striatum; SPm, spiny neurons in the matrix compartment of the striatum; ST, subthalamic sideloop; DA, DA neurons of the SN, pars compacta; PD, pallidal neurons; T, thalamic neurons.

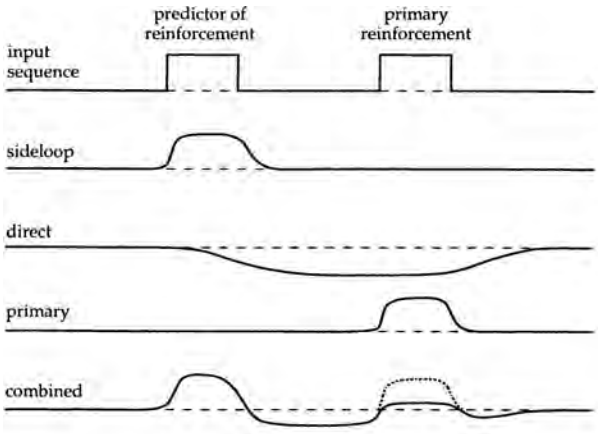


Figure 17. Hypothetical time course of signals generating DA neuron firing patterns. A pattern of excitatory signals which predicts reinforcement is delivered via projections from the cerebral cortical columns. The sideloop excitatory, direct inhibitory, and primary reinforcement signals converge on DA neurons, as shown in Figure 16. The bottom trace shows the combination of these three signals. The dashed curve shows the DA signal in presence of cocaine.

From this model, the appearance of a new predictive stimulus will further shift all sequence of events, always correlating the effective reinforcement (DAergic neuron discharge) to the very first conditioned stimulus or reinforcement predictor (Houk et al., 1995).

In view of the distinct functional role of striatal matrix vs striosomes it is interesting that the level of CREB phosphorylation is also different in these two subregions. It is of short duration in the matrix compartment and it is very persistent in striosomes as well as in other limbic striatal regions. This persistent phosphorylation can also support the neuroplasticity mediated by DA (Liu and Graybiel, 1998).

The adaptive critics-actor model, however, does not explain the response of DAergic neurons to any novel stimuli. An alternative theory explains such short-latency DA response as being associated with the reallocation of limited behavioral and cognitive processing capacity towards any unexpected event of behavioral significance, including reward (Redgrave et al., 1999). The caveat of this theory is, however, the absence of proved response of DAergic neurons to aversive stimuli. Finally, another group proposed the "spectral timing" model, in contrast to the temporal difference model, described before. According to this model, there are two distinct pathways for initial excitatory reward prediction (ventral striatum and PPTN) and for timed, inhibitory reward prediction (the striosomal cells) (Brown et al., 1999).

The adaptive critics-actor theory is particularly interesting and relevant for the present study because it allows us to explain the nature of compulsive drug use. Addictive drugs, for example psychostimulants, will directly activate the DAergic pathway, bypassing the routes taken by natural positive reinforcers (Figure 17). It will, therefore, escape the inhibitory regulation by "critics" unit, and thus any control of consumption will be lost. In addition, DA release will be extremely high, again compared to the natural situation.

In summary, striatum compartmentalization has three important functional consequences. First, its separation into striosome and matrix compartments has an important impact on striatal regulation of reward and motivation. Second, the two types of projections, striatonigral and striatopallidal, allow for differential processing of information in the BG, and for differential regulation of these two parallel ways by DAergic system. Finally, its compartmentalization into dorsal and ventral striatum, as a landmark for its motor and limbic division, is very relevant in terms of drug addiction. Drug reinforcing effects, caused by DAergic stimulation, will be additionally increased by the implication of limbic, emotional and motivational centers of the brain.

3. *Neurochemistry and genetics of drug addiction with special attention to psychostimulants*

Drug addiction is usually defined as compulsion to take a drug with loss of control over apparently voluntary acts of drug seeking and drug taking. The major task of the neuroscience of addiction is to understand the

mechanisms of transition which occurs between controlled and compulsive drug use. At the behavioral level the prolonged drug use is characterized by the phenomena of tolerance or habituation and sensitization which is described as an increment in response occurring upon repeated presentation of a stimulus. At the neuronal level drugs elicit two different types of responses: neuronal adaptations, i.e. homeostatic responses to excessive stimulation, and synaptic plasticity. These processes occur both on the cellular and system level and are paralleled by molecular changes residing on modified gene expression. Therefore, changes in gene expression correlate with the transition to an addictive state at the molecular level. Clearly the neuroanatomical substrate for this transition is the BG circuit supplied by midbrain DAergic inputs (Figure 18). All abused drugs ultimately react, directly or indirectly, with the mesolimbic DAergic pathway.

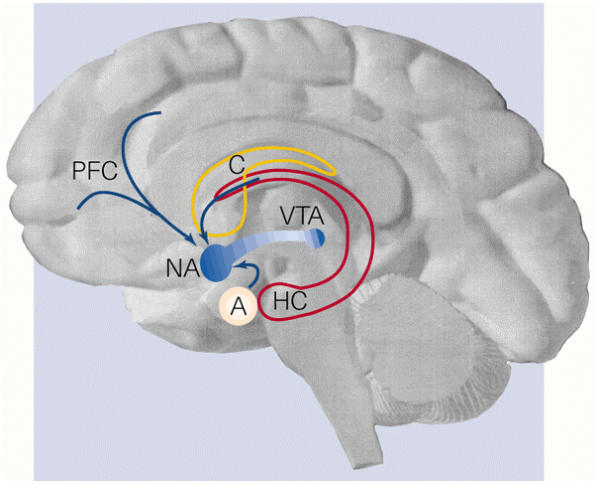


Figure 18. Major brain structures affected by drugs of abuse. The mesolimbic DA system originates in the ventral tegmental area (VTA) of the midbrain, and projects to the NAcc (NA). The amygdala (A), hippocampus (HC) and medial prefrontal cortex (PFC) sends excitatory projections to the NAcc. C, caudate nucleus (striatum) (adapted from (Robbins and Everitt, 1999)).

3.1. General overview of cocaine addiction

3.1.1. History of cocaine use

Cocaine is isolated from the plant called *Erythroxylon coca* (Figure 19). Probably the first users of cocaine were the native people of South America who used it by chewing coca leaves. A medical account of the coca plant was published in 1569. In 1860, Albert Neiman isolated cocaine from the coca leaf and described the anesthetic action of the drug when it was put on his tongue. Angelo Mariani, in the early 1880's produced a "healing" wine, called Vin Mariani, that contained 11% alcohol and 6.5 mg of cocaine in every ounce. Sigmund Freud, in 1884, recommended cocaine for a variety of illnesses and for alcohol and morphine addictions! Not surprisingly, many of his patients went on to become addicted to cocaine. In addition, the cases of fatal cocaine poisoning and alarming mental disturbances were reported. In 1886, John Pemberton developed Coca-Cola, a drink that originally contained cocaine and caffeine. Cocaine was removed from Coca-Cola in 1906. The Harrison Narcotic Act in 1914 made cocaine illegal in the US. Finally, in 1985, crack cocaine was introduced and rapidly became a major drug problem.

Figure 19. Erythroxylon coca plant.

3.1.2. Cocaine effects

Cocaine is a powerful psychostimulant that heightens alertness, especially to the sensations of sight, sound, and touch. It inhibits appetite and the need for sleep and provides intense feelings of pleasure. Paradoxically, it can make people feel contemplative, anxious, or even panic-stricken. In general, as for any other drug of abuse, the effects of cocaine depend on several factors, including the amount and the route of administration, the previous drug experience and the circumstances under which the drug is taken. Physical symptoms include accelerated heartbeat and breathing, and may induce convulsions and coma with higher doses .

Compared to amphetamine, cocaine has a very short duration of action that leads to a typical pattern of administration: rats or humans given access to adequate supplies of cocaine often binge, i.e., self-administer multiple closely spaced doses over long periods of time (Berke and Hyman, 2000).

With repeated administration over time, euphoria is gradually replaced by restlessness, extreme excitability, insomnia, and paranoia. Cocaine users usually do not develop tolerance to cocaine stimulant effects, but sometimes they report increased sensitivity to the drug's convulsant effects. This phenomenon may explain some deaths that occurred after apparently low doses.

Cocaine, as opposed to opioids and alcohol, does not cause severe physical dependence. However, it is perhaps the most powerful drug of all in producing psychological dependence. The heavy cocaine user suffers from severe depression upon drug withdrawal, and the risk of relapse is very high. However,

substantial variability is found in relation to individual's vulnerability to cocaine action, and the majority of occasional cocaine users do not become addicted (Addiction Research Foundation, 1995).

3.1.3. Neurochemistry of cocaine action on brain circuits

Psychostimulants act at axonal terminals of monoaminergic neurons as indirect monoamine agonists (Figure 20). Cocaine blocks the reuptake of DA by DAT, a member of the Na^+/Cl^--dependent twelve transmembrane domain transporter family, that also includes 5-HT and nor-adrenaline (NA) transporters. Amphetamine releases DA and reverses its transport via DAT (Hyman, 1996; Koob and Le Moal, 1997). The blocking of DAT in the striatum was classically considered the most important feature regarding the development of addiction. The rate at which DAT is blocked determines the perception of the "high" by a human user as determined by PET studies, whereas the level of blockade determines the intensity of this feeling (Volkow et al., 1999). It should be mentioned here, that other drugs of abuse also increase the DA concentration in NAcc, but by different, less direct routes. They can facilitate DA release by inhibition of GABAergic neurotransmission via mu-opioid receptors in the VTA in case of opioids and ethanol and via delta-opioid receptors in the NAcc in case of ethanol (Cowen and Lawrence, 1999).

The major DA receptors of the striatum are D1 and D2 receptors. D2 receptors are tonically stimulated by basal levels of DA, and this tonic activity is important for normal motor behavior, whereas D1 receptors are activated by phasic release of DA and are particularly relevant in terms of DA involvement in learning and addiction. However both receptors act synergistically, and moreover, the final effect of cocaine action depends on the co-activation of several systems. For example, the induction of IEG in the striatum requires both functional D1 and NMDA receptors (Torres and Rivier, 1993) and is blunted by the selective denervation of 5-HT projections under certain conditions (Bhat and Baraban, 1993), whereas 5HT-1B receptor knockout (KO) mice exhibit reduced expression of the IEG in the striatum (Lucas et al., 1997). Cocaine also acts as a local anaesthetic causing dose-dependent inhibitions of most spontaneously active and glutamate-stimulated neurons. These inhibitions which are not dependent on DA receptors may involve direct interaction with sodium channels (Kiyatkin and Rebec, 2000), but the precise mechanism is unknown.

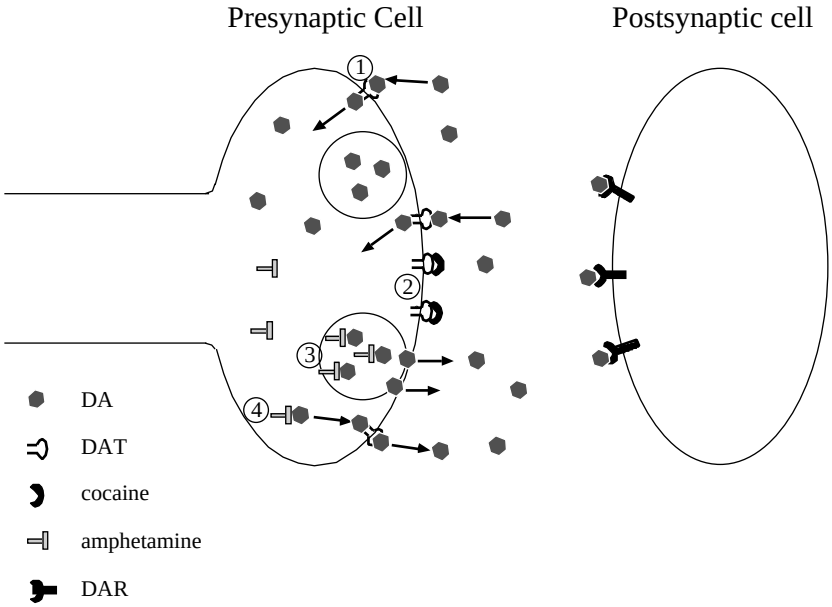


Figure 20. The psychostimulants action on DAergic neuron. In normal state DAT reuptakes DA from the synaptic cleft (1). Cocaine blocks DA reuptake sites (2), whereas amphetamine displaces DA from vesicles (3) and reverses the transport of DA by DAT (4). The net output in case of both drugs is the increase of DA concentration in the synaptic cleft and over-stimulation of DA receptors (DAR).

3.2. Experimental approaches for drug addiction study and their relative impact on our understanding of drug action

3.2.1. Classical electrophysiological and psychopharmacological methods

Several areas of the brain are known to be the targets of intracranial self-stimulation. One of these regions is the medial forebrain bundle, a collection of fibers, by which the DAergic input reaches the forebrain targets (Figure 21). Acute administration of drugs that are abused in humans increases the reward value of intracranial self-stimulation, and drug withdrawal decreases this value (Koob, 1998; Kreek and Koob, 1998; Leshner and Koob, 1999), suggesting that continuous drug use produces the state of reward deficit. Many studies have implicated the NAcc in drug action. Rodents will self-infuse a variety of drugs of abuse (e.g. amphetamine, morphine, phencyclidine and cocaine) into the NAcc, primarily in the shell region (McBride et al., 1999). The shell of the NAcc is particularly sensitive to D1 receptor

antagonists (Caine et al., 1995), suggesting a particular way of cocaine action via the D1 receptors. Extracellular DA is increased in the NAcc upon lever pressing for food, upon self-stimulation of the lateral hypothalamus, and upon injection of amphetamine or cocaine into the NAcc, as measured by microdialysis (Hernandez and Hoebel, 1988). Some NAcc neurons exhibit changes of the firing rate synchronized to the response-contingent delivery of water or cocaine. Interestingly, the activity in response to cocaine is influenced by the stimulus context in which the drug was delivered (Carelli and Deadwyler, 1997). This context-dependent drug action depends at least partially on the stress state of the animal, i.e. on the state of activation of the hypothalamic-pituitary-adrenal (HPA) axis and is important not only for the establishment of drug addiction, but also for the expression of withdrawal syndromes. Intracerebroventricular administration of a corticotropin-releasing factor (CRF) antagonist reverses the anxiogenic-like responses following withdrawal from chronic cocaine and ethanol administration (Koob, 1999).
Psychopharmacological studies have pointed out the role of the VTA in the development of sensitization. Injection of opioids or psychostimulants directly into the VTA leads to the development of sensitization (Kalivas and Stewart, 1991). This mechanism may involve protein kinase C (Steketee et al., 1998). Injection of a D1 receptor antagonist into the VTA blocks the development of neurochemical (DA release) but not behavioral (motor-stimulant response) sensitization to cocaine (Steketee, 1998).

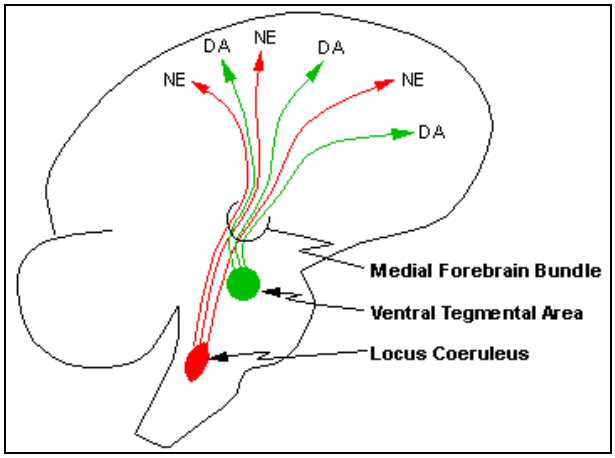


Figure 21. Medial Forebrain Bundle

3.2.2. Genetic methods

The development of genetics and of molecular biology techniques opened a new page in the study of drug addiction. One direction of research aims at finding the genetic factors responsible for the development of drug abuse. A second line of experiments studies gene expression in different animal models of drug intake in order to dissect the role of different gene products in the development of the distinct temporal and functional phases of drug addiction. The conclusions drawn from both types of studies are then tested in transgenic animal models, including KO mice.

3.2.2.1. Classical genetic studies

It was clear for a long time that a genetic component is implicated in drug addiction. Family, as well as twins or adoption studies provided a lot of information clarifying the relative impact of genetic and environmental factors on drug abuse. However, human studies have obvious limitations. Both ethical considerations and the unique and highly complicated genetic background of every single person make the search for genetic factors extremely difficult. The first animal tools for dissecting the particular genes and their chromosomal locations important for drug action, appeared with the development of inbred mouse strains. Inbred strains are populations of animals produced by more than 20 generations of crossings within closely related animals, usually sisters and brothers (Crabbe and Belknap, 1980). It is postulated that these animals are homozygous at all gene loci. About 20 inbred strains of mice exist, and many of them show clearly distinct behavior in respect to various addictive drugs. The inbred strains are useful for studying the effects of a particular mutation on a specific trait, as well as for correlation studies allowing the mapping of different traits to a common genetic determinant. Inbred strains maintain a stable genetic background during many years, allowing for high reproducibility and contingency in genetic studies.
One important application of inbred strain technology has been the development of recombinant inbred strains. In this technique different inbred strains are crossed, and then various F2 generations are mated together to result in an entire panel of new inbred strains, each with different combination of parental genes. Then a particular trait, e.g. reinforcing effects of cocaine, is studied across these strains, followed by the localization of this trait to a chromosomal locus (quantitative trait locus) by means of traditional linkage analysis. Current methods allow the determination of a narrow chromosomal region. Subsequent database searches result in the identification of candidate genes within that subregion. The final phase of this positional cloning approach will be the testing of candidate genes in transgenic models. Searching for quantitative trait loci linked to cocaine addiction resulted in at least three putative loci, one of these containing the gene for the 5-HT_{1B} receptor (Crabbe et al., 1999).

3.2.2.2. Studies of gene expression

Studies of gene expression can be divided into two groups:

1) "target-directed" studies by immunohistochemical or RNA-based methods, such as Northern blot, *in situ* hybridization etc., which require previous identification of a gene product of interest;
2) random searching for new genes important for a particular function. In this case the control and experimental situations are compared (e.g. drug treatment), and high throughput screening for differentially expressed genes is performed. Methods used include subtractive hybridization, differential display, microarrays or DNA chips.

The differential screening of cDNA libraries was a fuse in a subsequent burst of methods allowing for identifying differentially expressed genes (Rebagliati et al., 1985; St John and Davis, 1979). In this technique, two cDNA libraries, isolated from cells to be compared (control and experiment), were immobilized on solid support and hybridized with radioactively labeled cDNA probe, representative of total mRNA population of the cells of interest. The method was very time-consuming and required a large amount of starting material. It was substantially improved by the introduction of subtractive cDNA libraries, where only presumably differentially expressed sequences are present. This method was especially useful when the differentially expressed RNAs are of low to moderate abundance in the cells in which they occur (Sargent and Dawid, 1983). A great variety of protocols of subtractive hybridization was developed and successfully applied. However, the obvious disadvantage of this technique lies in the fact that it is impossible to compare more than two mRNA samples at once.

Later on, two PCR-based methods for screening of differential expression were developed. In the first method, arbitrarily primed PCR fingerprinting of RNA, the arbitrarily selected primer was used at low stringency for first and second strand cDNA synthesis, followed by PCR amplification of reaction products (Welsh et al., 1992). The second method, discussed later in more details was differential display (Liang and Pardee, 1992). PCR-based methods have several technical advantages, compared with hybridization-based ones. They are much quicker, allow for simultaneous detection of several groups of differentially, both up- and downregulated genes, only small amount of mRNA (less than 5 µg) is required, and sensitivity is very high. They are also simple and easy in use.

Finally, a third group of methods was developed, based on comparison of restriction pattern between the samples, such as serial analysis of gene expression (Velculescu et al., 1995), restriction landmark cDNA scanning (Suzuki et al., 1996) or restriction endonuclease-based gene expression fingerprinting (Ivanova and Belyavsky, 1995). However, the relevance of these intellectual efforts in large part loose their merit with the appearance of cDNA microarrays and similar high throughput techniques, which will replace completely other methods of differential gene expression screening. This technology however is introduced slowly in neuroscience research, given the fact that the availability of arrays, representative of the full palette of genes important for brain function, especially for rat brain, is still low.

In this work, the method of differential display was modified and used, therefore this procedure will be described in details. The first step of the differential display protocol is a reverse transcription with primers consisting of a poly(T) stretch and one or two more additional 3’ bases, which anchor the primers to the poly(A) tail of mRNAs. The second step is a low stringency PCR reaction with the same primer and also with another short arbitrary primer which will anneal at different positions relative to the first primer. These annealing positions are randomly distributed in distance from the poly(A) tail. Therefore, the amplified products from various mRNAs differ in size. These PCR products are labeled with radioactive deoxyadenosine triphosphate, and can then be displayed on an acrylamide gel. The majority of expressed mRNAs will be represented if twelve possible 3’ primers and twenty 5’ primers are used in all combinations for independent amplifications and displays. A band of interest, such as one produced by one kind of cell versus another, can be excised from the gel, its cDNA reamplified by PCR, cloned and then sequenced. Its sequence can then be compared with data banks and used to isolate longer sequences from DNA libraries, and ultimately studied for function. The method is very powerful, but it has some problems, including the number of false positives generated during 40 rounds of low stringency PCR, the need for cloning in order to analyse the differentially expressed signal and sequence heterogeneity of the bands excised from acrylamide gel. Therefore in the last years many authors undertook attempts to improve and facilitate the use of the method. Some of them concerned the design of primers for PCR. These included the addition of restriction sites to 5’ends of the primers (Zhao et al., 1995), the design of gene family specific primers ("targeted differential display") (Donohue et al., 1995; Joshi et al., 1996) and the use of non-radioactively labeled primers (Chen and Peck, 1996). Other modifications concerned new strategies for the identification of true positive candidates, such as restriction-enzyme fingerprinting approach (Shoham et al., 1996), plasmid dot-blotting to screen cloned cDNA fragments with cDNA PCR products as probes (Callard et al., 1994), partial sequencing of cDNA inserts (Zhao et al., 1996), single-strand conformation polymorphism of cDNA inserts (Mathieu-Daude et al., 1996; Zhao et al., 1996) and screening of multiple clones from differential display by the technique of mRNA hybridization using a slot blot (Liu and Raghothama, 1996). These modifications substantially facilitated the use of the method. Differential display studies already contributed to the progress of different areas of neuroscience's. For example, it provided some cues on genes important for sleep-waking cycle (Cirelli and Tononi, 1999), psychiatric illness related to cognition, memory and mood (Flanigan and Leslie, 1997), auditory plasticity (Lomax et al., 2000), long-term potentiation (Matsuo et al., 1998; Matsuo et al., 2000) and it will certainly gain more place in brain research in the next years.

Genes, whose expression has been found to be regulated by cocaine, are summarized in Table 1 and some examples are described below. Some of the changes were found at the protein level (immunohistochemistry, Western blot etc.) which very often correspond to transcriptional regulation of a particular gene, but may also reflect posttranscriptional, or even posttranslational modifications (for example, the increase in TH immunoreactivity upon chronic cocaine treatment, see Table 1 for details). Changes, occurring both at the

mRNA and at the protein level, are often detected in different cellular compartments, the mature protein being transported away from the cell body. The protein-containing compartment can be localized in very different brain regions, at a long distance from the initial site of mRNA synthesis.

3.2.2.2.1 *Acute studies*

A suitable marker of neuronal activity is the expression of IEG, the first ones to be activated after a particular treatment. Important IEG for brain function belong to zinc finger (zif268, alternative names NGFI-A or egr1) and to leucine zipper (fos/jun) transcription factor families. Their expression following drug application was extensively studied, in particular c-fos activation upon increase in cAMP concentration brought about by the binding at several neurotransmitter receptors.

Acute cocaine or amphetamine treatment results in an increase in mRNA concentrations of c-fos and other transcription factors, as well as Fos-like immunoreactivity in the dorsal striatum and NAcc (Graybiel et al., 1990; Hope et al., 1992). Fos expression is immunocytochemically detectable after 1h, reaches its maximum after 2h, and is absent 48h after treatment, thus showing a typical pattern of IEG expression. c-fos induction in the striatum has different patterns of expression in striosome-matrix compartments and limbic subdivisions of the striatum (Graybiel et al., 1990). These differences are drug-specific. Cocaine induces IEG expression in cells distributed broadly through the striatum, whereas amphetamine-specific expression is highest in rostral striosomes (Graybiel et al., 1990; Moratalla et al., 1996; Moratalla et al., 1992). Interestingly, the same pattern of induction was shown for another IEG, arc (activity-regulated, cytoskeletal-associated gene), the particularity of which is its expression in dendrites (Tan et al., 2000). This drug-specific pattern may account for different behavioral effects of the two drugs (Canales and Graybiel, 2000). Cocaine-related c-fos induction is limited to striatal neurons expressing SP (Kosofsky et al., 1995) and the protein phosphatase inhibitor DARPP-32, but not Enk (Berretta et al., 1992). Concerning accumbal subdivisions, Fos-like immunoreactivity upon acute cocaine is higher in the shell than in the dorsolateral striatum, and lowest in the core (Barrot et al., 1999). The effect is mediated by D1 receptor (Young et al., 1991), at least in part by NMDA-sensitive receptors (Torres and Rivier, 1993) and by 5HT-3 receptor (Humblot et al., 1998). The intracellular cascade leading to c-fos activation is shown in Figure 22.

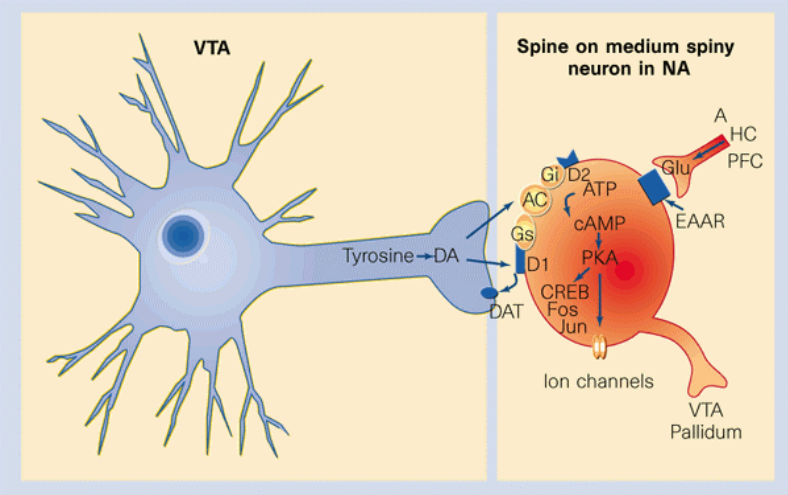


Figure 22. Neural systems of addiction. A DA-releasing neuron from the ventral tegmental area (VTA) is shown innervating a dendritic spine of a medium spiny neuron in the nucleus accumbens (NA). The DA transporter (DAT) is the main site for cocaine action, which inhibits the re-uptake of DA by the VTA neuron, where it is initially produced. DA acts at two types of DA receptors (D1 and D2), coupled to guanine-nucleotide-binding proteins (Gs and Gi respectively), which modulate adenylyl cyclase (AC) and cAMP-dependent protein kinase (PKA). Possible substrates for PKA include ion channels and the nuclear transcription factors CREB, Fos and Jun. Neurons in the NA in turn receive glutamatergic inputs from the amygdala (A), the hippocampus (HC) or the prefrontal complex (PFC). EAAR, excitatory amino-acid receptor; Glu, glutamate (adapted from (Robbins and Everitt, 1999)).

The expression of c-fos is not limited to BG, it has also been found in many limbic areas, including the cingulate cortex, the claustrum, the piriform cortex, the lateral septal nucleus, the paraventricular nucleus of the thalamus, the lateral habenula, and the amygdala (Brown et al., 1992). In the thalamic paraventricular nucleus the effect was mediated by the D3 DA receptor (Deutch et al., 1998). The composition of the AP-1 complex and requirements for its activation may be different in different regions, e.g. acute cocaine induces both c-fos and jun-B mRNA expression in the striatum, but only c-fos in the cerebellum (Couceyro et al., 1994). Studies of c-fos expression allowed dissecting out of many effects of cocaine, with an aim to understand the relative involvement of different brain regions and the importance of particular neurotransmitter systems. Studies of peptidergic identity of cocaine-responsive neurons allowed for a model of DA-opioid peptide

interaction. However, little is known about genes, whose expression is regulated by c-fos, and their relative impact in drug action. The development of c-fos KO animals gave first insights in this subject.

Studies showed that zif268 expression often paralleled that of c-fos. Both acute cocaine and amphetamine led to zif268 induction in the striatum, both dorsal and ventral (Bhat et al., 1992; Moratalla et al., 1992). Again, the pattern of expression was different for striosomal and matrix compartments in rostral striatum. The effect is mediated by D1 receptor and by the 5-HT system (Bhat and Baraban, 1993).

Although the expression of both c-fos and zif268A is mediated by D1 DA receptor, the concomitant activation of D1 and D2 receptors acts to intensify their expression. High-affinity neurotensin receptors are likely to be involved in this D1-like/D2-like cooperative action. Pretreatment with neurotensin receptor antagonists prevented the IEG expression induced by both acute cocaine and D1/D2 receptors agonists treatment in cells expressing Enk mRNA (Alonso et al., 1999).

Data about acute c-jun expression are more controversial. Some studies show its induction in the NAcc (Hope et al., 1992), but other researchers could not confirm this in the striatum (Moratalla et al., 1993). The controversy is probably due to the variations of expression in different striatal subregions.

c-fos expression is NMDA-receptor sensitive. This finding led to extensive studies of changes in expression of Glu receptors subunits upon cocaine treatment. Various subunits were expressed in differential fashion upon acute cocaine (Table 1 (Ghasemzadeh et al., 1999). These changes could be important in terms of cocaine action on a network level because they may alter excitatory neurotransmission in the mesocorticolimbic DA system, which could play a significant role in the enduring biochemical and behavioral effects of cocaine. This hypothesis is strengthened by the fact that chronic cocaine also alters the relative expression of different Glu receptor subtypes (see Table 1).

Cocaine-induced IEG expression is different in three mice strains (Table 1) (Kuzmin and Johansson, 2000), yielding to robust, slight, or even complete absence of expression. These differences in gene expression pattern parallel the different cocaine reinforcing properties for the three strains and show the importance of consistency in drug studies if one aims at finding cause-effect relationships in drug addiction, and especially the importance of choosing the right strain for transgenic studies.

Many genes are regulated by psychostimulants, but only a small portion have been studied in terms of the functional significance of such changes, and consequently, of the role of a particular protein in drug addiction. For example, acute cocaine or amphetamine leads to rG_{β_1} (rat G-protein β-subunit) up-regulation in the striatum and the prefrontal cortex, in particular in the shell of the NAcc (Wang et al., 1997) as identified by subtracted differential display. The dose-response curve for amphetamine in the striatum showed the persistence of expression from 2 to 48 hrs. Antisense oligonucleotide treatments that attenuate rG_{β_1} expression in regions including the NAcc abolish the establishment of behavioral sensitization. These treatments do not alter acute behavioral responses to cocaine, confirming the specific function of this protein for sensitization phenomenon.

Another protein important for the development of sensitization is neurotrophin-3 (NT-3). Acute cocaine causes a transient increase of NT-3 mRNA in the VTA, whereas intra-VTA microinjections of NT-3 leads to a sensitized behavioral response to a subsequent cocaine challenge (Pierce et al., 1999). The effects of NT-3 on the initiation of behavioral sensitization are mediated by activation of the Ras/MAP kinase signal transduction system.

Another example of a regulated protein found by differential display is CART (cocaine- and amphetamine- regulated transcript). Acute cocaine or amphetamine treatment increases CART expression in the striatum in a region-specific manner (Douglass et al., 1995). Subsequent studies showed that CART plays a major role in the regulation of feeding behavior, whereas the function of CART in relation to drug addiction is still unknown (Kuhar and Dall Vechia, 1999; Thim et al., 1998). The studies of cocaine-induced CART expression, however, underlined the importance of the experimental protocol used in drug addiction studies, because other investigators could not reproduce the up-regulation of CART in the striatum (Fagergren and Hurd, 1999). The failure to reproduce these findings probably comes from the slightly different protocol used by subsequent authors (see Table 1). In general this problem of the protocol is not very pronounced in the case of acute studies, the majority of protocols consisting of a single injection with an established dose of psychostimulant followed by 1 or 2 h before brain extraction. However, the protocol problem is much more pronounced and critical in chronic and sensitization studies.

Studies of gene expression also showed the interference of cocaine with hormonal function. The majority of studies concerned stress-related hormones. Drugs of addiction and stress are mutually-interfering factors. The stressors facilitate the acquisition of drug self-administration, probably by increasing the reinforcing efficacy of drugs of abuse (Piazza and Le Moal, 1998). Drugs, in turn, modulate the release of stress hormones. Cocaine, for example, alters hypothalamic and extrahypothalamic/limbic CRF concentrations (Sarnyai, 1998). Acute cocaine treatment increases CRF transcription in the paraventricular hypothalamic nucleus (Zhou et al., 1996). Similar reciprocal interaction exists between cocaine and oxytocin / vasopressin (VP) systems. For instance, oxytocin facilitates, whereas VP inhibits the development of behavioral sensitization to cocaine, while cocaine in turn induces dose- and region-specific changes in oxytocin and VP contents in the hypothalamus and limbic structures (Sarnyai, 1998).

3.2.2.2.2 *Chronic and self-administration studies*

c-fos induction by cocaine is highest in the dorsal striatum, a region with lowest basal level of Dyn expression. On subsequent treatment days the induction of c-fos goes down, whereas Dyn expression in this region gradually increases. Thus, both the regional and temporal patterns of gene expression display an inverse relationship between Dyn and c-fos expression. This observation led to the hypothesis that Dyn may "blunt" the cocaine induction of c-fos and that its expression in BG serves to prevent the neurons of the direct striatal pathway from excessive D1 stimulation. This hypothesis is strengthened by the finding that c-fos expression can be inhibited by an agonist of kappa-receptor, on which dynorphin normally acts. The activation of the Dyn system could also contribute to the dysphoric syndrome associated with cocaine dependence (Koob, 1996). The expression of both c-fos and dynorphin may be regulated by CREB phosphorylation upon the activation of D1 receptor (Figure 22). CREB interacts with functional regulatory elements in both the c-fos and prodynorphin genes, and is phosphorylated in response to DA in a D1-dependent manner (Hyman et al., 1995). The drug-induced expression of Dyn is also specific for striosomes-matrix compartments of the striatum. Following chronic apomorphine treatment Dyn expression is increased preferentially in striosomal neurons (Gerfen et al., 1991). As opposed to Dyn, changes in SP parallel the changes in c-fos activation (Gerfen et al., 1998; Steiner and Gerfen, 1993) and are not specific for any compartment.

Following chronic apomorphine treatment Enk expression remains unchanged (Gerfen et al., 1991), however cocaine self-administration causes a slight increase in Enk expression in the NAcc (Hurd et al., 1992).

Chronic cocaine administration leads to the desensitization of both c-fos and zif268 induction in the forebrain, caudate putamen, and NAcc (reviewed in (Torres and Horowitz, 1999)). Interestingly, after the return of mRNA and protein concentrations to basal levels, an increase in AP-1 binding activity is observed which persists upon chronic cocaine treatment (Hope et al., 1992). The increase in AP-1 binding in the NAcc and striatum develops gradually over several days and remains at 50% of its maximal levels seven days after the last cocaine exposure. The AP-1 complex contains at least four "chronic" Fos-related antigens (FRAs), some of which display delta FosB-like immunoreactivity (Hope et al., 1994; Mumberg et al., 1991). They are different from delta FosB, because they respond to chronic cocaine, and heterodimerize primarily with Jun-D and Jun-B, as opposed to c-Jun. Some other chronic treatments may also induce chronic Fras (Chen et al., 1995), which may therefore serve as a marker for long-lasting synaptic activity, like c-fos for acute neuron activation. Upon cocaine, chronic FRAs are expressed preferentially in Dyn-expressing projection neurons (Moratalla et al., 1996). Altogether, FosB, along with c-fos, fra-1 and fra-2, comprise the fos family of transcription factor proteins. FRAs are differentially regulated by DA receptors and include two types of proteins: those independent of D1 receptor activation and those dependent on D1 receptors for expression following drug challenge (Moratalla et al., 1996). Studies in organotypic slice cultures of newborn rat striatum have shown the induction of Fos-like and Fra-like proteins upon D1 receptor stimulation. Fos-like proteins were induced mainly in striosomes, and Fra-like proteins in striatal matrix as well (Liu et al., 1995).

In summary, chronic cocaine exposure leads to time-varying alterations in the inducibility of leucine-zipper transcription factors in individual neurons and long-lasting network changes in which groups of striatal neurons express these proteins, suggesting that the behavioral sensitization may reflect an enduring functional reorganization of BG circuits (Moratalla et al., 1996). It is not known if other IEG, such as zif268, have several stable isoforms, similar to FRAs, or whether their expression is definitively shut down upon repeated exposure to a drug.

A small number of genes, regulated by cocaine, have been studied in terms of their role in drug addiction development, for example NAC-1, a transcription factor named because of its selective up-regulation in the NAcc three weeks after cocaine self-administration (Cha et al., 1997). Further studies have shown that NAC-1 induction may down-regulate postsynaptic DA transmission, thus minimizing the behavioral impact of cocaine administration (Kalivas et al., 1999). In addition, adenoviral-mediated overexpression of NAC-1 in the rat NAcc prevented the development but not the expression of behavioral sensitization to cocaine (Mackler et al., 2000).

Table 1 summarizes other changes in gene expression upon chronic cocaine under various protocols. In the absence of a standard protocol, it is difficult to obtain reproducible and reliable results, as shown in studies of TH and DAT changes upon chronic cocaine. Different groups undertook such an investigation, given the role of both proteins in DA turnover, with diverse results (Table 1). Besides the protocol of drug administration itself, other factors need to be carefully evaluated, such as animal strain, age, sex, etc. For example, Kuzmin and coll. investigated self-administering cocaine mice and used not saline-receiving animals, as usual, but yoked controls, receiving cocaine passively, as controls for the neurochemical effect of non-contingent cocaine infusion. This showed differences in the expression of several genes between cocaine- and saline-treated animals, as well as between mice receiving contingent and non-contingent regimens of cocaine treatment (Table 1). This and other studies show the urgent need for the elaboration of more standard and precisely defined protocols of drug treatment in order to obtain consistent and comparable data. However it is doubtful to expect such a standardization in the nearest future. The temporary solution is to use the same protocol in frame of one or several groups (for example, acute "binge" protocol in Table 1) throughout the years.

Table 1. Changes in gene expression induced by cocaine treatment

Protocol	Changes	References and Remarks
Acute		
5-50 mg/kg	Induction of c-fos and zif268 in striatum, both dorsal and ventral	(Barrot et al., 1999; Berretta et al., 1992; Bhat and Baraban, 1993; Bhat et al., 1992; Graybiel et al., 1990; Humblot et al., 1998; Kosofsky et al., 1995; Moratalla et al., 1992; Torres and Rivier, 1993; Young et al., 1991)
	Increase in c-fos, c-jun, fosB, junB, and zif268 mRNAs and in Fos-like immunoreactivity in the NAcc	(Hope et al., 1992)
	No induction of c-jun in striatum, but c-fos and junB (peak at 1 hr)	(Moratalla et al., 1993)
	Induction of c-fos and junB in striatum, only c-fos in cerebellum	(Couceyro et al., 1994)
	Induction of c-fos in limbic areas (cingulate cortex, claustrum, piriform cortex, lateral septal nucleus, paraventricular nucleus of the thalamus, lateral habenula, and amygdala) and in BG (dorsomedial striatum and NAcc)	(Brown et al., 1992; Deutch et al., 1998)
2 mg/kg	Increased zif268 and decreased secretogranin II mRNAs in the caudate putamen in C57 mice	(Kuzmin and Johansson, 2000); These effects were more widespread in DBA and absent in the 129 mice.
25 mg/kg	Robust transient increase in arc mRNA levels in striatal neuronal cell bodies and dendrites (2 hrs after injection)	(Fosnaugh et al., 1995; Tan et al., 2000); suppressed by D1 receptor blockade, reserpine treatment, or striatal 6-OHDA lesions
	Reduction in mRNA level for GluR3, GluR4, and NMDAR1 subunits. Reduction of NMDAR1 mRNA level in dorsolateral striatum and VTA. GluR2 mRNA level was not changed in any brain regions examined.	(Ghasemzadeh et al., 1999)
	$rG\beta_1$ (rat G-protein β-subunit) up-regulation in striatum and prefrontal cortex 4 hrs after the injection	(Wang et al., 1997)
	Transient increase in NT-3 mRNA in VTA	(Pierce et al., 1999)
20 mg/kg i.p. 3 x 15 mg/kg 1 hr apart	CART increase in striatum 1 hr after the injection Up-regulation of prodynorphin mRNA in the caudate putamen, but not in the NAcc; CART up-regulation in the medial accumbens shell of females, and in central amygdala of male rats, no regulation in striatum! (all 1 hr after the last injection)	(Douglass et al., 1995) (Fagergren and Hurd, 1999)
	In striatum and NAcc: increase in proenkephalin mRNA (only in striatum), decrease after 24 and 48 hrs. Decrease in D2 receptor mRNA after 3 hrs, return to basal level after 24 hrs and increase after 48 hrs	(Przewlocka and Lason, 1995)
20 and 40 mg/kg, i.p.	Decrease of D2 receptor in OT, but not in the striatum or the NAcc	(Spyraki and Sealfon, 1993)
	Alteration in hypothalamic and extrahypothalamic/limbic CRF concentrations	(Sarnyai, 1998); dose- and time-dependent
5 mg/kg i.v. or two sequential sc injections of 40 mg/kg	Increase in steady-state levels of CRF mRNA	(Rivier and Lee, 1994)
	Changes in oxytocin and VP contents in the hypothalamus and limbic structures	(Sarnyai, 1998); both dose- and region-selective
15 mg/kg	Decrease in prepro-thyrotropin-releasing hormone mRNA in the amygdala and hippocampus (45 min postinjection)	(Sevarino and Primus, 1993); no changes in the hypothalamus, NAcc, or thalamus

	Striatal and nigral increase in neurotensin-like immunoreactivity	Alburges and Hanson, 1999, Brain Res, Feb 6;818(1): 96-104; blocked by ibogaine, psychoactive indole alkaloid
	A transient increase in synaptotagmin IV mRNA in the dorsal striatum (1 hr after injection)	(Denovan-Wright et al., 1998); drug-, region- and time-specific
30 mg/kg	A transient and dose-dependent induction of the expression of a brain plasticity-related molecule, tissue plasminogen activator (tPA) mRNA with its peak at 3 h postinjection in a group of neurons of the medial and insular prefrontal cortices that project to the medial striatum, and the piriform cortex	(Hashimoto et al., 1998); the induction of tPA mRNA could be implicated in an initial step in the plastic rearrangements in the neuronal circuits underlying long-lasting changes in behavioral expression
Acute "binge"		
3 consecutive doses of 15 mg/kg i.p., at hourly intervals for 3 days	Increase in CRF mRNA levels in the hypothalamus and amygdala on day 1, return to base-line levels on day 2, decrease on day 14 but only in hypothalamus, return to base-line level after 10 days of withdrawal Increase in preproenkephalin, preprodynorphin mRNAs in caudate-putamen Increase in preprodynorphin mRNA in the caudate-putamen, decrease in kappa-opioid receptor mRNA in the SN but not in caudate-putamen No change in DAT mRNA in SN or VTA Increase in mu-opioid receptor mRNA in frontal cortex, NAcc, and amygdala, but not in the caudate-putamen, thalamus, hippocampus, and hypothalamus (30 min after the last injection of single day of treatment)	(Zhou et al., 1996) (Spangler et al., 1996) (Spangler et al., 1996) (Maggos et al., 1997) (Yuferov et al., 1999); D1-receptor dependent mechanism
3 consecutive doses of 15 mg/kg i.p., at hourly intervals for 2 days	Kappa-opioid receptor mRNA down-regulated in VTA and NAcc (30 min after the final injection)	(Rosin et al., 1999); In the VTA, more pronounced decrease in combination with ethanol, in the NAcc with only cocaine
Chronic		
	Suppression of Fos-like immunoreactivity accompanied by long-lasting increase in AP-1 binding in NAcc and striatum. This AP-1 complex contains at least four FRAs, some of which display delta FosB-like immunoreactivity	(Hope et al., 1994)
Twice daily with 15 mg/kg for a total of 10 injections	Widespread suppression of basal zif268 mRNA levels in rat forebrain between 8 and 24 hr after the last cocaine injection	(Bhat et al., 1992)
Every 8 min for 6 h per day with 0.33 mg/infusion i.v. for 7 days (similar to self-administration pattern)	Increase in TH mRNA (45 and 50% in SN and VTA respectively)	(Vrana et al., 1993)
	Increased TH immunoreactivity by 30-40% in VTA only	(Beitner-Johnson and Nestler, 1991)
Two doses (15 or 30 mg/kg i.p.) for 6 days to produce behavioral sensitization to a challenge either 24 hr (early withdrawal) or 3 weeks (late withdrawal) later	At early withdrawal, after both doses, TH immunoreactivity in the VTA increased to 125% of saline controls by 24 hr after the cocaine challenge, with no changes in TH mRNA levels. At the late withdrawal, TH immunoreactivity or mRNA in the VTA were not altered 24 hr after a saline or cocaine challenge	(Sorg et al., 1993); the time course of TH expression in the VTA parallels previously reported changes in somatodendritic DA release and autoreceptor desensitization
Twice daily with 15 mg/kg for 6,5 days	Greater than 40% decrease in nigral DAT mRNA	(Xia et al., 1992); not 4 hrs after acute injection, nor after repeated treatment followed by a 72-h withdrawal period

Once daily with 10, 15 or 25 mg/kg i.p. for 8 days or twice daily with 25 mg/kg i.p. for 8 days	DAT mRNA decrease in both SNc, and VTA (1 hr after the last injection)	(Letchworth et al., 1997; Letchworth et al., 1999); no difference in DAT protein or binding sites within the striatum
Three doses at 1 h intervals each day with 15 mg/kg i.p. for 14 days (chronic "binge" pattern) or 10 days withdrawal from a chronic “binge” pattern	No change in DAT mRNA in SN or VTA	(Maggos et al., 1997)
2 weeks of schedule similar to self-administration pattern followed by ten days of withdrawal	Decrease in DAT in the interfascicular and caudal linear nuclei (project to the NAcc), but not in other DAergic midbrain cell groups	(Cerruti et al., 1994)
“Binge” cocaine	Serotonin transporter decrease within the dorsolateral dorsal raphe, DAT decrease within the SNc and parabrachial pigmentosis, NA transporter increase within the locus coeruleus	(Burchett and Bannon, 1997); correlational analysis indicated that post-cocaine levels of DAT, 5-HT and NA transporters mRNAs were not associated with cocaine-induced sensitization
Once daily with 20 mg/kg i.p. for 10 days followed by 14 d withdrawal?	Increase in D1 and D2 receptor mRNAs in different brain structures	(Schmidt-Mutter et al., 1999); similar to gamma-hydroxybutyrate effect
20 mg/kg i.p. for 15 days	Increase in D2 receptor mRNA in OT, but not in the striatum, 24 hrs but not 7 days after withdrawal.	(Spyraki and Sealfon, 1993)
Repeated	Proenkephalin mRNA increase after 3hrs, depletion after 24 and 48hrs, D2 receptor mRNA: decrease after 3hrs, no changes after 24 or 48hrs	(Przewlocka and Lason, 1995)
Once daily for 10 days	30, but not 10 or 20 mg/kg increased the expression of preprodynorphin, but not preproenkephalin mRNAs	(Daunais and McGinty, 1994)
50 mg/kg per day, for periods between 24 and 336 hrs	Mu-opioid receptor increase in NAcc (develops 2 days after exposure, peaking at 3 days, returned to baseline after 4 days)	(Azaryan et al., 1998)
Repeated cocaine	Long-lasting but reversible reduction in neuropeptide Y protein and mRNA levels in cerebral cortex and NAcc	(Wahlestedt et al., 1991); prevented by lesion of medial prefrontal cortex
3 weeks after discontinuing 1 week of daily injections (15-30 mg/kg i.p.)	Reduction of the CluR3 mRNA level. Increased level of GluR2 mRNA in prefrontal cortex. Increase of mGluR5 mRNA levels in nucleus accumbens shell and dorsolateral striatum	(Ghasemzadeh et al., 1999); the GluR2 mRNA level was not changed in any other brain regions examined
Cocaine withdrawal	Alterations of CRF concentration in the hypothalamus, amygdala, and basal forebrain	(Sarnyai, 1998)
	Changes in oxytocin and VP contents in the hypothalamus and limbic structures	(Sarnyai, 1998); both dose- and region-selective
Twice daily with 15 mg/kg for 14 days	Marked regulation in the NAcc, hypothalamus, amygdala, and hippocampus	(Sevarino and Primus, 1993); regulation was strongly dependent on the length of cocaine withdrawal and persisted up to 72 h postinjection in the amygdala
Once daily with 15 mg/kg i.p. for 10 days	Increase in neurotensin mRNA in the shell of the NAcc, in the posterior dorsomedial striatum in the ventrolateral striatum and in the fundus striati (one hour after the last injection)	(Betancur et al., 1997)

	Decreased level of phosphoRet, the protein kinase that mediates GDNF signaling, in the VTA	(Messer et al., 2000)
	Up-regulation of cytochrome oxidase subunit I (but not III or IV) mRNA in the NAcc (33%) and caudate-putamen (35%)	(Walker and Sevarino, 1995); cytochrome oxidase activity was increased in the NAcc and the medial prefrontal cortex, but not in the caudate-putamen
Self-administration		
Intravenous cocaine self-administration in mice versus yoked control animals, receiving cocaine passively	An increase in c-fos mRNA in lateral and basolateral amygdala in active cocaine-receiving animals, decrease in yoked controls receiving cocaine. In caudate putamen, both cocaine regimens increased c-fos mRNA. Non-contingent cocaine infusions increased zif268 mRNA expression in the core of the NAcc, medial caudate putamen and frontal cortex, whereas self-administration eliminated these effects. In the core of the NAcc and piriform cortex there was increased, and in the medial amygdala decreased secretogranin II mRNA in yoked controls compared with saline controls. In contrast, in basomedial and central nuclei of the amygdala, increased secretogranin II mRNA was found in self-administering mice.	(Kuzmin and Johansson, 1999)
Variable self-administration for a 7 day period	Dyn and SP elevation, greater in the dorsal striatum. Slight elevation of Enk in NAcc	(Hurd et al., 1992)
Self-administration for four weeks	D1 receptor mRNA increase in the forebrain, both D1 and D2 - in limbic region, one week after return to baseline	(Laurier et al., 1994)
Self-administration	NADH dehydrogenase (26% decrease in the NAcc, 305% increase in the ventral midbrain, no changes in the caudate putamen, changes in hypothalamus and cerebellum)	(Couceyro et al., 1997); mitochondrial gene expression, identified by differential display
3 weeks of i.v. self-administration	NAC-1 increase exclusively in the NAcc 3 weeks after last injection	(Cha et al., 1997)

3.2.2.3. Transgenic animals

The development of transgenic and, in particular, KO mice models allowed for a more precise understanding of the function of different proteins in drug addiction. A lot of attention was paid to KO of genes directly involved in DA transmission, i.e. genes coding for DAT, DA receptors and DARPP-32.

DAT KO mice display spontaneous hyperlocomotion despite major adaptive changes. DA persists at least 100 x longer in the extracellular space, and the psychostimulants had no effect on locomotor activity (Giros et al., 1996). Even if they do not express DAT, the primary target for cocaine, mice still acquire cocaine self-administration, although this takes longer than in wild-type animals (Miller et al., 1999). This could be attributed to a role of the serotonin transporter in cocaine addiction, but it is not clear whether such a role exists in normal animals or if this is part of an adaptation processes in KO mice. It is likely, however, that serotonin plays a role in normal effects of cocaine, because 5-HT1B KO mice display a markedly reduced effect of cocaine on c-fos induction in different brain structures, mostly in the striatum (Lucas et al., 1997).

Individual differences in DAT expression may underline differences in human substance abuse vulnerability. Interestingly, mice with 20-30% striatal DAT overexpression display enhanced reward conferred by cocaine (Donovan et al., 1999).

D1-receptor deficient mice have proven the critical role of this receptor in psychostimulant action. The cocaine-induced hyperactivity was abolished in these animals, as well as cocaine-induced inhibition of action potential generation in the NAcc (Xu et al., 1994). Cocaine or amphetamine were not able to induce c-fos, JunB and zif 268, or to regulate Dyn expression. Cocaine however caused the abnormal increase of SP expression in the striatum (Drago et al., 1996). Only D1-mediated effects were abolished, because haloperidol, a neuroleptic acting preferentially on D2 receptors, remained effective in inducing catalepsy and striatal Fos/Jun expression. This psychostimulant action, therefore, does not implicate receptor synergy (Moratalla et al., 1996). KO animals did not exhibit sensitization to cocaine (Xu et al., 1994).

Mice lacking DA D4 receptors were hypersensitive to ethanol, cocaine, and metamphetamine (Rubinstein et al., 1997).

DARPP-32 KO mice showed heightened SP-like immunoreactivity in the striatum, but no increase of DeltaFosB by chronic cocaine was observed. The mutants showed a higher rate of locomotor sensitization to repeated cocaine exposures, but the effect of acute cocaine on locomotor activity was unchanged (Hiroi et al., 1999). KO mice failed to show a significant elevation of either plasma ACTH or corticosterone levels following 'binge' cocaine, suggesting that DARPP-32 could mediate the stimulatory effect of cocaine on the HPA axis (Zhou et al., 1999).

Other models have been developed, over-expressing intracellular proteins implicated in cocaine action.

Over-expression of CREB in NAcc decreased the rewarding effects of cocaine and made low doses of the drug aversive whereas Dyn expression was increased. Overexpression of dominant-negative mutant CREB increased the rewarding effects of cocaine and decreased the expression of Dyn. Blockade of kappa opioid receptors antagonized the negative effect of CREB on cocaine reward (Carlezon et al., 1998). This points out the role of Dyn in the development of cocaine tolerance.

Mice lacking the fosB gene show abnormal biochemical and behavioral responses to chronic administration of drugs of abuse or antidepressant treatments (Nestler et al., 1999). In contrast, transgenic mice with inducible deltaFosB expression in the subset of NAcc neurons in which cocaine induces the protein display increased responsiveness to the rewarding and locomotor-activating effects of cocaine. These effects of deltaFosB are mediated partly by induction of the AMPA glutamate receptor subunit GluR2 in the NAcc. These results support a model in which deltaFosB, by altering gene expression, enhances sensitivity to cocaine and may contribute to cocaine addiction (Kelz et al., 1999).

However the evaluation of data from transgenic, in particular KO animals, must be done carefully. First, in mutant animals, substantial compensation occurs during development . The more necessary the deleted gene, the more profound compensations will occur in the case of viable mutants, so more errors will be possible during evaluation of experimental results. The use of conditional KO provides more relevant results. Second, the strategy of producing KO should be carefully considered, especially when performing behavioral tests. KO animals in most cases have a mixed genotype between the strain at the origin of the ES cell line and the second strain used for breeding the chimeric animals. Very often these strains have completely a different phenotype in behavior, for example, in respect to memory tests or drug dependence (Crawley et al., 1997; Deroche et al., 1997). For example, two common strains for KO production, C57BL/6J and 129/J mice, have significant differences in relation to the effects of "binge" cocaine administration on stereotypy and locomotor activity (Schlussman et al., 1998). Therefore, care should be taken to purify as much as possible such mixed strains by consequent breeding and testing for the presence of mutation. Finally, this situation is further complicated by the fact that historically the great majority of behavioral, lesions and electrophysiological tests were performed in rats, where the protocols are well established, and molecular biology is more studied in mice. Even if these two rodents are highly similar in terms of gene homology, this is not at all the case in terms of behavior and even neurochemistry. One of the most flagrant examples is a surprising insensitivity of rats to MPTP, a neurotoxin specific for the DAergic system, as opposed to mice, non-human primates or humans. The reasons of such a resistance remain a mystery (Miller et al., 1999).

3.3. Associative and non-associative mechanisms of drug addiction

The acute reinforcing effects of cocaine and amphetamine lead to patterns of drug use that, in epigenetically vulnerable individuals, result eventually into addiction, a state hypothesized to be the result of plastic changes in multiple neural circuits. Many of these changes are caused by the induction of IEG following drug treatment (Figure 23). If the drug treatment is repeated, these changes then enchain downstream molecular events, accounting for long-lasting neuroadaptations which could exert significant behavioral consequences in the longer term.

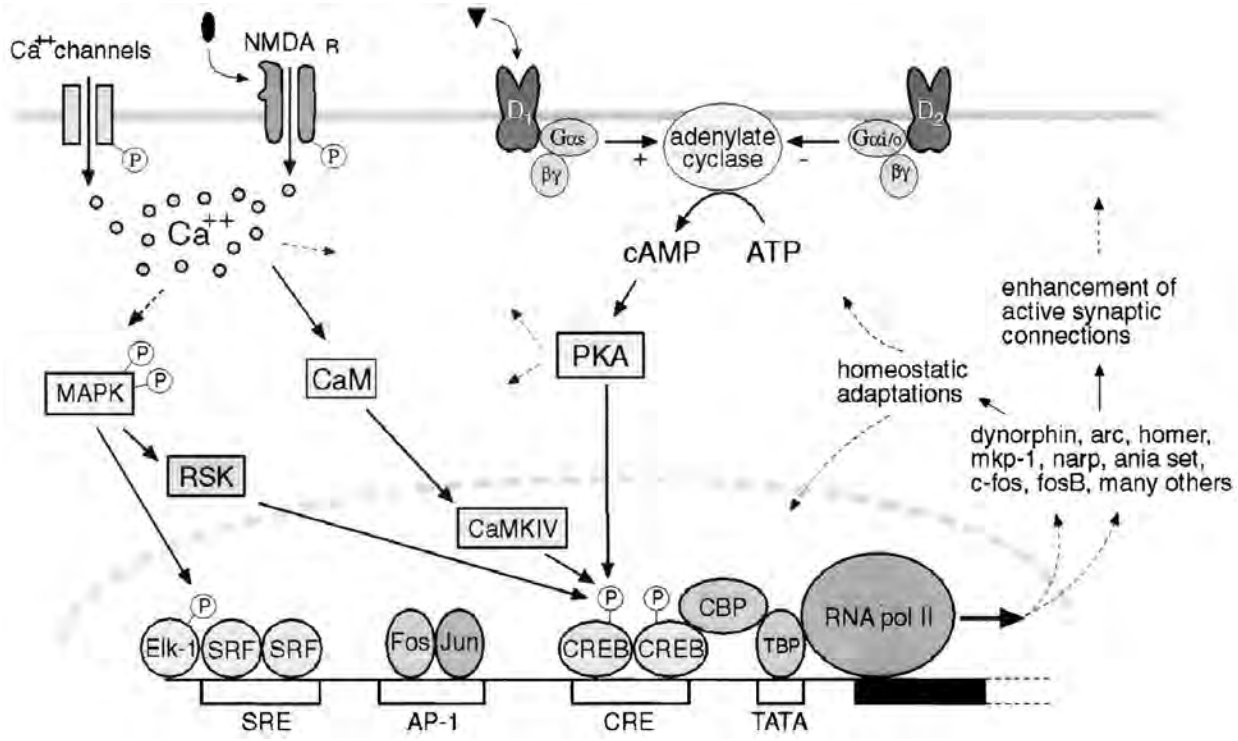


Figure 23. Induction of IEG expression is under the joint control of calcium- and cAMP-dependent signal transduction pathways. In the striatum these pathways appear to be mutually inhibitory at many stages (not shown), but their effects inside the nucleus can be cooperative. Both PKA and CaMK-IV can phosphorylate CREB at serine 133. Calcium-dependent CREB phosporylation may also occur as a result of activation of the ERK/MAPK in striatal cells. ERK/MAPK also increase the transcription of striatal IEGs through phosphorylation of the transcription factor Elk-1. A complex set of genes can be induced in striatal neurons. Some genes are part of a homeostatic response, reducing sensitivity to subsequent stimulation; others may be involved in consolidating changes in the strength of specific synaptic connections. Abbreviations: D1, DA D1 receptor; D2, DA D2 receptor; PKA, cAMP-dependent protein kinase; CaM, calmodulin; CaMKIV, calcium/calmodulin-dependent protein kinase IV; MEK, MAP and ERK kinase; MAPK, mitogen-activated protein kinase; SRF, serum response factor; AP-1, activator protein-1; CRE, cAMP response element; CREB, CRE binding protein; TBP, TATA binding protein; RNA pol II, RNA polymerase II (adapted from (Berke and Hyman, 2000)).

Plasticity changes that underlie addiction can be divided conceptually into three groups:

1) compensatory adaptations in neural systems that regulate autonomic and other somatic functions leading to physical dependence and withdrawal;
2) adaptations in the mesoaccumbens brain reward circuitry itself resulting in the emotional and motivational aspects of dependence and withdrawal;
3) synaptic plasticity, which allows for the association of drug-related stimuli with specific learned behaviors (Berke and Hyman, 2000; Hyman, 1996).

The first mechanism is not very important for psychostimulants, because, unlike the opiates and ethanol, cocaine and amphetamine do not produce physical dependence; they are, nonetheless, among the most reinforcing and addictive drugs known, underscoring the importance of plasticity in emotional circuits (Hyman, 1996). Neuronal adaptations account for both tolerance and some types of sensitization whereas synaptic plasticity is important for specific forms of sensitization related to associative learning.

3.3.1. Tolerance

The development of tolerance is explained in Figure 24. Prolonged drug use produces profound adaptive changes acting to maintain equilibrium by reducing drug effects (tolerance), and acute withdrawal will again disturb this novel homeostasis and produce negative physical and emotional symptoms. Manifestations in humans include dysphoria, anhedonia, and drug craving with eventual reinstatement of drug use. Anhedonia in humans may be modeled in rats by transient decreases in DA neurotransmission and elevations in electrical brain self-stimulation reward thresholds observed after cocaine administration (Markou and Koob, 1991).

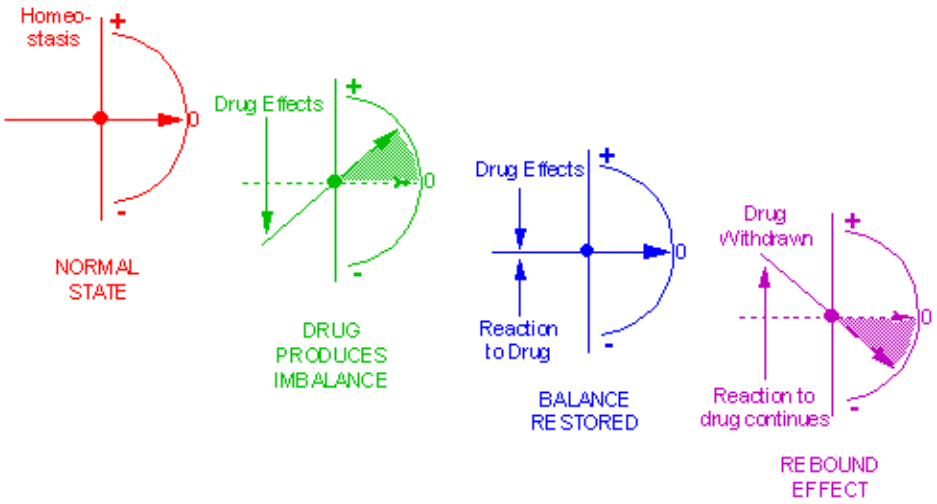


Figure 24. The development of tolerance. Continuous exposure to a drug disturbs the homeostasis of the mesolimbic DAergic system in its normal state. The system therefore undergoes substantial adaptations to restore its balance by reducing drug effects (tolerance). Nevertheless, it is clear that the balance is only possible in the presence of the drug, therefore abstinence will unmask neural adaptations that have occurred, and a subset of these may produce withdrawal symptoms generally opposite to those of the drug (adapted from (Timmons and Hamilton,)).

Some cellular and molecular events leading to tolerance development, are shown on Figure 25 and represent compensatory adaptations to excess DA stimulation which result in down-regulation of DA transmission. First, acute cocaine treatment results in the phosphorylation and internalization of D1 receptors, accounting for “acute tolerance” phenomenon (Figure 25) (Dumartin et al., 1998; Roseboom and Gnegy, 1989; Tiberi et al., 1996). Second, cocaine and amphetamine induce expression of prodynorphin mRNA and Dyn peptides in striatonigral neurons (Jaber et al., 1995) which signal back on presynaptic DA terminals within the striatum via recurrent axon collaterals (Figure 25). Acting via kappa receptors on DA terminals, Dyn decreases DA release (Steiner and Gerfen, 1995). Indeed, kappa receptor agonists produce an aversive dysphoric syndrome in drug naive humans and rats, analogous to the state induced by drug withdrawal in drug users. Third, both cocaine and morphine increase levels of TH, while decreasing the levels of neurofilament proteins in the VTA (Beitner-Johnson et al., 1992). Thus, despite the increased TH gene transcription, the axonal transport of the mature protein will be slowed down, leading to decreased DA synthesis in the presynaptic space (Figure 25).

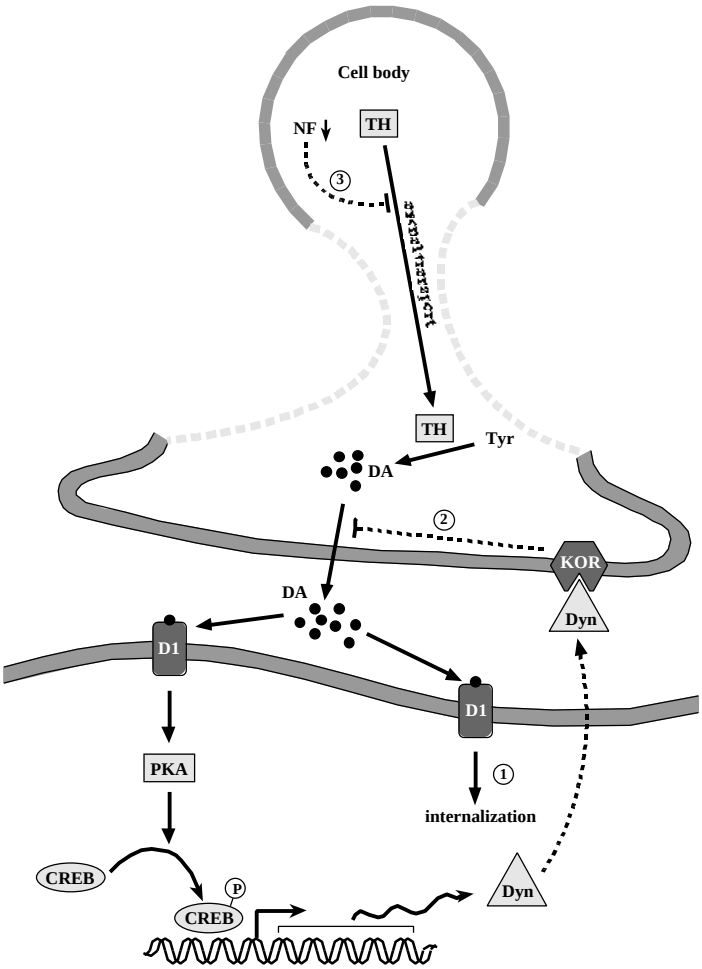


Figure 25. Cellular mechanisms of tolerance. Acute cocaine treatment yields into the internalization of D1 receptors (1). Chronic cocaine induces the expression of Dyn which, by acting on presynaptic kappa opioid receptors (KOR), inhibits DA release (2). Chronic cocaine also decreases the synthesis of neurofilament proteins (NF), leading to decreased axonal transport of tyrosine hydroxylase (TH) to the presynaptic terminal (3). Tyr, tyrosine.

When drug administration ceases, the neural systems which have undergone neuroadaptations, gradually return to their normal sensitivity. This can take anywhere from minutes to weeks depending on the particular homeostatic response, but so far none appears sufficiently long-lasting to be involved in the persistent tendency of addicted individuals to relapse (Berke and Hyman, 2000).

3.3.2. Sensitization

Sensitization is more likely to occur with intermittent exposure to a drug, in contrast to tolerance, which is more likely to occur with continuous exposure (Koob, 1996). Neural changes responsible for these apparently opposite phenomena might however coexist in the same system. By applying different protocols of drug treatment, it is possible to induce first sensitization, then tolerance, and vice versa (Dalia et al., 1998). Tolerance, being a more pronounced and short-lasting phenomenon, is masking the expression of sensitization.

3.3.2.1. Context-independent sensitization

Context-independent sensitization relates to a sensitized response to non-specific cues such as stress or drug exposure, leading eventually to relapse to drug use. This might be due to neuroadaptations, as in the case of tolerance. For example, chronic cocaine is known to induce profound modifications in the HPA axis, which would explain the phenomenon of stress-induced sensitization.

Other manifestations of context-dependent sensitization are explained by changes in the mesoaccumbens circuitry that would increase the effects of DA. These can be achieved, for example, by a decreased sensitivity of presynaptic DA D2 autoreceptors and supersensitivity of postsynaptic D1 receptors. In fact, after a period of psychostimulant administration, the ability of a subsequent dose to evoke dopamine release in the striatum can be increased (for reviews, see (Kalivas and Stewart, 1991; Robinson and Becker, 1986)). This effect can persist for at least several weeks (Hooks et al., 1994; Kalivas and Duffy, 1993; Kalivas and Duffy, 1993; Robinson et al., 1982). The enhancement of D1 receptor responses in the NAcc was also reported in correlation with locomotor sensitization to cocaine (Henry and White, 1995). This effect can persist for one month after withdrawal. These changes do not correlate with an increase of the D1 receptor number or affinity, but likely reflect modifications in post-receptor signal transduction. In fact, chronic treatment of rats with cocaine produced increases in cyclic AMP-dependent protein kinase activity and decreases in Gi_ and Go_ levels in the VTA, NAcc, and locus coeruleus (Nestler et al., 1990; Terwilliger et al., 1991). Changes in transcription factors expression upon chronic cocaine

could also explain some types of neuroadaptations, e.g. the switch from c-fos to chronic FRAs, which leads to a different composition of the AP-1 transcription activation complex and therefore to a different set of expressed genes. However, there is no evidence to date for long-lasting up- or downregulation of mRNA or protein which may account for the persistence of some forms of sensitization—and, in humans, of addiction (Berke and Hyman, 2000).

3.3.2.2. Context-dependent sensitization

Compensatory adaptations (changes in neurotransmitter release, postsynaptic sensitivity, etc.) are restored rapidly with discontinuation of drug administration. The symptoms of withdrawal diminish, but the relapse to drug use persists for years. In addition to adaptations within the brain reward circuitry itself, drugs induce a set of molecular mechanisms leading to long-lasting changes in the synaptic efficacy and structural synaptic change (Berke and Hyman, 2000). At the behavioral level it is expressed by production of long-lived, positively biased, emotional memories that may be activated by environmental or interoceptive cues. Relapse to drug use in humans often occurs when addicts encounter people, places, or other cues associated with their prior drug use (Childress et al., 1986; Shiffman et al., 1996). Context-dependent sensitization and cue-conditioned human relapse suggest that the brain stores specific patterns of drug-related information, resulting from prior associative learning. Humans and animals can readily learn to take addictive drugs; this process requires the specific recognition of drug-associated cues and the performance of specific actions. Drugs act as very effective positive reinforcers (Woolverton, 1992). In addition, cues associated with drug administration acquire motivational significance; for example, rats will choose to spend more time in a location in which they have passively received an injection of psychostimulants than in another location paired with saline injection ("conditioned place preference"; (Tzschentke, 1998)).

DA plays a role as a reinforcement learning signal in the striatum (Wickens and Kötter, 1995) which involves the timely-organized coactivation of corticostriatal (Glu-ergic) and DAergic circuits, resulting in the reinforcement of particular synapses. At a behavioral level this means an association between a particular set of stimuli and a particular behavioral response. The involvement of these striatal "habit"-learning mechanisms by addictive drugs could explain the tendency for drug-related cues and contexts to provoke specific behaviors, such as drug self-administration (Robbins and Everitt, 1999; White, 1996). The activity of cortical areas may also account for the observation that giving an amphetamine injection in a novel environment greatly enhances both striatal IEG induction and behavioral sensitization, without affecting striatal dopamine release (Badiani et al., 1998).

The development of stimulus–response habits has been attributed particularly to learning processes involving dorsal parts of the striatum. Facilitation of synaptic plasticity in the ventral striatum may also contribute to drug use through enhanced learning related to the motivational significance of drug-related cues (Carr and White, 1983). The changes in synaptic plasticity first involve the activation of transcription factors, such as CREB, arc, etc. Later phases may engage also structural changes related to synaptic connections, similar to the formation of new synaptic contacts in the late phase of hippocampal LTP (Engert and Bonhoeffer, 1999). In fact, chronic amphetamine administration causes increased dendritic spine density in the NAcc, as well as an increased number of branched spines (Robinson and Kolb, 1997). DA can stimulate neurite extension and growth cone formation in embryonic striatal cultured neurons through a pathway that involves D1 receptors, the cAMP/PKA pathway, and protein synthesis (Schmidt et al., 1996; Schmidt et al., 1998). Addictive drugs can induce synaptic plasticity or associative learning in abnormal fashion. Reinforcing events that are fully predicted do not evoke DA release and hence do not provoke further learning (see § II.2. C.3) (Schultz, 1998). However, the direct pharmacological actions of psychostimulants and other addictive drugs may override such normal constraints on learning (Di Chiara, 1998). The continuous DA release could lead to excessive strengthening of synaptic patterns representing drug-taking behavior, relative to other behaviors performed by the animal, thus narrowing the behavioral repertoire towards drug-related activities (Koob et al., 1998).

In addition, the manner of drug intake becomes progressively more fixed—particular sequences of actions become "ritualized" and automatic (Tiffany, 1990), consistent with the involvement of the dorsal striatal "habit"-learning system. In summary, these learning-related actions of drugs are primarily responsible for the compulsive nature of drug use.

Non-associative and associative mechanisms of sensitization are not mutually exclusive. Molecular events leading to both types of sensitization may occur in the same cell. For example, two major neuroadaptations caused by chronic cocaine are the increased expression of the GluR2 subunit, following FRAs induction and the decreased sensitivity of calcium channels (Kelz et al., 1999; White et al., 1998). Later on both modifications could contribute to the changes in synaptic plasticity associated with LTP. Thus, nonassociative changes in dopamine neurotransmission may set the thresholds for the effects of drugs on associative learning.

Neurotrophins also contribute to changes in the mesoaccumbens system. There is a feedback loop, whereby drugs of abuse decrease signaling through the GDNF pathway in the VTA, which then increases the behavioral sensitivity to subsequent drug exposure (Messer et al., 2000). NT-3 contributes to the initiation of behavioral sensitization to cocaine by activating the Ras/MAP kinase signal transduction system (Pierce et al., 1999). Data about BDNF are more controversial. BDNF KO heterozygous mice show a delayed development of locomotor cocaine sensitization (Horger et al., 1999). BDNF enhances the initial

stimulant effects of cocaine and facilitates the development of sensitization to repeated cocaine doses (Horger et al., 1999). However, BDNF also produces a progressive augmentation in behavioral activation with repeated drug administration with no changes upon subsequent cocaine treatment (Pierce et al., 1999). These results are in agreement with the observation that BDNF elicits an increase in the phosphorylation of extracellular signal regulated kinase (ERK) and thereby prevents an additional increase in response to drug exposure (Berhow et al., 1996).

The neurobiology of dependence is a highly complex phenomenon, consisting of both single and repeated exposure to drugs (where rewarding, discriminative and aversive properties of a drug are important) under environmental and genetic conditions that lead to or are accompanied by drug-seeking behaviors (Stolerman, 1993). Environmental conditions include the hormonal status of the organism. For example, glucocorticoids may contribute to the state-dependent activation of mesencephalic DAergic neurons and thus to the individual vulnerability to the drugs of abuse (Piazza et al., 1996). Gender differences in susceptibility to addiction are also very important. In female rats the gonadal hormones estrogen and progesterone modulate DA activity in the striatum. Estrogen is able to enhance DA release and DA-mediated behaviors (Becker, 1999).

3.4. Cocaine and neural axon remodeling

3.4.1.1.1 *Cocaine and cytoskeletal proteins*

Psychostimulants produce extensive biochemical adaptations in brain monoamine, especially DA, systems. They also may alter the morphology of neurons in different brain regions, including that receiving monoaminergic innervation. Treatment with either amphetamine or cocaine (but not general motor activity) increase the number of dendritic branches and the density of dendritic spines on medium spiny neurons in the shell of the NAcc, and on apical dendrites of layer V pyramidal cells in the prefrontal cortex. Cocaine also increases dendritic branching and spine density on the basilar dendrites of pyramidal cells. Changes in dendritic architecture may modify the patterns of synaptic connectivity in the NAcc and prefrontal cortex and account for some of the persistent neurobehavioral consequences of repeated exposure to psychostimulant or other addictive drugs (Robinson and Kolb, 1999). In fact, experimental animal studies showed a reduction in shape and size of mesolimbic dopaminergic neurons after chronic morphine administration (Pezawas et al., 1998).

Several reports show the interaction of psychostimulants with cytoskeletal proteins. As was mentioned before, cocaine administration induces several IEG, which code for transcription factors. In addition, it affects expression of a recently identified "effector" IEG referred to as arc (activity-regulated, cytoskeleton-associated). Arc is a protein with homology to spectrin that is associated with the actin cytoskeleton. Both striosome and matrix striatal neurons show arc upregulation, which is mediated by D1 dopamine receptor. Immunohistochemical studies indicated that Arc induced by cocaine is expressed in neuronal cell bodies and dendrites. Thus, Arc may be involved in structural alterations underlying neuronal plasticity triggered by cocaine (Fosnaugh et al., 1995; Tan et al., 2000).

Cocaine also affects the metabolism of tau which stabilizes microtubules and promotes microtubule assembly. In fact, cocaine treatment decreases tau in the cytoplasm as well as the membrane fraction of cultured human neuroblastoma cells (Lew, 1992).

There is an increasing amount of data about the importance of neurofilament proteins in drug addiction. An initial observation showed that chronic administration of either morphine or cocaine decreases levels of the three neurofilament proteins, NF-H, NF-M, and NF-L, by between 15% and 50% in the VTA. The regulation is selective and region-specific. No detectable changes were observed in the levels of eight other major cytoskeletal or cytoskeletal-associated proteins analyzed and neurofilament levels were not altered in the SN, or other parts of the brain or spinal cord. Furthermore, the decline in neurofilament expression was not observed following chronic treatment with psychotropic drugs without reinforcing properties, or chronic stress (Beitner-Johnson et al., 1992). Later it was shown, that chronic ethanol and nicotine exposure as well as "unpredictable" stress also decreased levels of immunoreactivity of neurofilament proteins in the VTA but not in the SN, in parallel with increased levels of TH and glial fibrillary acidic protein immunoreactivity (Bunnemann et al., 2000; Ortiz et al., 1995; Ortiz et al., 1996). Finally, the strains of alcohol-preferring versus nonpreferring rats display different levels of neurofilaments in the VTA, strongly favoring the hypothesis that neurofilaments expression may be related to preference for different drugs of abuse (Guitart et al., 1993).

In confluent endothelial monolayers, cocaine induced a rapid increase in membrane permeability, a marked disruption of F-actin and the formation of intercellular gaps (Kolodgie et al., 1999).

In addition to its direct effects on cellular morphology, psychostimulants interfere with the changes induced by growth factors. Cocaine inhibits neuronal differentiation in a dose- dependent fashion, in NGF-stimulated PC12 cells, without affecting cell viability. Dopamine and GBR 12909, a potent DAT inhibitor, yield similar effect, which is mediated by D1 receptors (Zachor et al., 2000). Interestingly, both stimulating effects of NGF and inhibiting effects of cocaine are mediated by the same intracellular message, c-fos. NGF induces a transient peak of c-fos expression, and induction of late genes expression, leading to the neuronal phenotype. In the cocaine-treated cells, c-fos level persisted up to several days (Zachor et al., 1998).

3.4.2. Cocaine and development

Drugs of abuse modify signaling of neurotransmitters and intracellular messengers. These same neurotransmitters regulate various aspects of cell proliferation, survival, migration, circuit formation and

establishment of topography during central nervous system development (Levitt, 1998). Non-surprisingly, the exposure to cocaine *in utero* leads to profound alterations in brain development.
Rabbits exposed to cocaine *in utero*, show a marked decrease in both dendritic bundling and typical long, straight MAP2-stained profiles in the anterior cingulate cortex. The organization of the apical dendrites is modified. In cocaine-treated offspring, the dendrites coursed in an irregular, wavy manner throughout the layers, suggestive of dendrites that are longer than normal, although cortical thickness is unchanged. The drug-induced modification of cortical regions development is not uniform, as suggested by lack of dendritic changes in visual cortex (Jones et al., 1996). *In vitro* results confirm the involvement of cocaine in brain development. Both the number and length of neurites were dramatically reduced by cocaine in cocultures of neurons and glial cells from mouse embryonic brain. These changes preceded extensive neuronal death. Scanning electron microscopy demonstrated a shift from a multipolar neuronal pattern towards bi- and unipolarity prior to the rounding up and eventual disappearance of the neurons. Selective toxicity of cocaine on neurons was paralleled by a concomitant decrease of the culture content in microtubule-associated protein 2. In contrast, cocaine did not affect astroglial cells and their glial fibrillary acidic protein content (Nassogne et al., 1998; Nassogne et al., 1995). Finally, in developing GABAergic neurons cocaine produced cytotoxic effects that were expressed in drastic decrease in the number of neurons and in degeneration of their processes (Glezer et al., 1999).
Recent studies show that cocaine is also able to modify the expression of developmentally important molecules in adult brain. EphB1 and ephrin-B2, a receptor and ligand of the Eph family, are candidate guidance molecules for the development of mesostriatal and mesolimbic dopaminergic projections. EphB1 and ephrin-B2 are expressed in complementary patterns in the midbrain dopaminergic neurons and their targets, and the ligand specifically inhibits the growth of neurites and induces cell loss of SN, but not VTA, dopaminergic neurons. In addition, ephrin-B2 expression is upregulated by cocaine and amphetamine in adult mice, suggesting that ephrin-B2/EphB1 interaction may play a role in drug-induced plasticity in adults as well (Yue et al., 1999).
Eph receptors and their ligands appear to guide axons by repelling the growth cone, rather than attracting it (Orioli and Klein, 1997). They are similar in this aspect to another group of axon guidance molecules, the semaphorins. Both Sema3A and ephrin A5 induce physiologic growth cone collapse by actin filament rearrangements and alterations in membrane dynamics (enhanced endocytosis) (Fournier et al., 2000). Interestingly, besides their role in axon guidance, semaphorins are expected to have multiple functions in morphogenesis and tissue remodeling by mediating cell-repelling cues through plexin receptors (Artigiani et al., 1999). This feature makes semaphorins an attractive candidate as modulators of dopaminergic neurons plasticity in adult brain, for example, following cocaine treatment. Sympathetic neurons undergoing dopamine-induced apoptosis, show up-regulation of secreted collapsin-1 (Sema3A) and collapsin response mediator protein. A synchronized induction of mRNA preceded commitment of neurons to apoptosis. Dopamine-induced apoptosis was prevented by blocking collapsin signaling. It has been suggested, that, before their death, apoptosis-destined neurons may produce and secrete destructive axon guidance molecules that can affect their neighboring cells and thus transfer a "death signal" across specific and susceptible neuronal populations (Shirvan et al., 1999). It is tentative to imagine, that these *in vitro* apoptotic properties of semaphorins may be represented *in vivo* by more subtle specific modifications of neuronal morphology and synaptic rearrangements.

3.4.2.1. Aims of this work

In summary, cocaine treatment results in substantial neuroadaptations and synaptic plasticity in the mesoaccumbal DAergic pathway (Berke and Hyman, 2000). Both phenomena require gene expression and protein synthesis for their expression. A number of neuroadaptations already known and the acquired knowledge allows us to presume that this mechanism plays a minor role in the long-lasting relapse to drug taking, determining the addiction phenomenon. Synaptic plasticity might instead be a crucial factor in this respect. Some data exist about modified synaptic efficiency and connections in the brain, repeatedly exposed to a drug, however very little is known about the factors responsible for such modifications. Recent findings implicate Ephrin-B2, an axon guidance molecule, in drug addiction. Ephrin-B2 is a guiding molecule playing the major role in the establishment of mesostriatal DAergic projections during development, and it is also induced in the NAcc upon “binge” cocaine treatment with a peak at 24 hrs after drug withdrawal (Yue et al., 1999). This intriguing finding suggests that the very same neurotrophic factors, normally required for the development of the DAergic system, might be involve in structural changes of DAergic neurons in drug-induced plasticity. At the same time this very protocol, resulting in Ephrin-B2 over-expression, may serve as model of a set-point for induction of structural plasticity in the mesoaccumbens pathway. However, it is important to have a representative picture of gene expression changes at this point in order to understand the nature and the functional significance of such changes. We therefore decided to perform a screening for differentially-expressed genes at this particular time-point by means of differential display of mRNA. Two modifications of the method were introduced allowing first for increased reproducibility and easy handling of the method, and second for a gene-specific screening for the semaphorins, members of another family of axon guidance molecules. In addition, new rat semaphorins were cloned by RT-PCR using degenerate family-specific primers.

II. METHODS

1. *Differential Display of mRNA (according to (Brenz Verca et al., 1998))*

This modified protocol (based on the original differential display procedure (Liang and Pardee, 1992)) uses longer primers, designed to increase the specificity and to permit direct sequencing and ribonuclease protection assay with re-amplified fragments.

1.1. Reverse transcription:

1) Denature and anneal 0.2-2 µg of total or polyA RNA with oligo dT set (Table 2), 2.5 µM, for 5 min at 75 ˚C, then cool down to 0°C
2) Add the following components: RT Buffer, 1x; DTT, 10 mM; dNTPs, 625 µM; RNAsin, 40 U (optional!); MMLV-RTase, 400 U). Total reaction volume 40 µl.
3) Incubate for 1 hr at 37 ˚C, then heat for 10 min at 75°C.
4) Precipitate with EtOH, resuspend in TE buffer.

Alternative (no need for precipitation):

1. Heat mix of primers, 1µM; and RNA, 0.2-3 µg; for 5 min at 65 ˚C, then put at 37 ˚C, let some minutes.
2. Add: RT Buffer, 1x; DTT, 10 mM; dNTPs, 20 µM; RTase, 200 U. Total reaction volume 20 µl. Incubate for 1 hr at 37 ˚C, then for 5 min at 80 ˚C.

1.2. PCR:

1. Mix the following components: PCR Buffer, 1x; MgCl2, 1 mM; dNTPs, 2 µM; {α-^{32}P}-dATP, 2 µCi; Taq DNA Pol, 1.5 U; oligo dT primer or pool, 1 µM; arbitrary primer (Table 2), 1 µM; RT-mix, 2 µl.
2. Run the following thermocycler profile:

94°C, 2 min		initial denaturation (facultative)
94°C, 1 min 40°C, 2 min 72°C, 1 min	}	5 cycles
94°C, 45 sec 56°C, 1 min 72°C, 45-60 s	}	35 cycles
72°C, 5 min		final extension

1.3. Electrophoresis:

1. Mix 7 µl of sample with 4 µl of loading dye, incubate for 2 min at 80 ˚C, chill on ice and load 4 µl on gel (6%). Electrophoresis for about 3 Hr at 60 W constant power until the xylene canole dye runs to within 10 cm from the bottom.
2. After electrophoresis, transfer the gel directly onto a piece of Whatman 3MM paper. Cover the gel with a sheet of Saran wrap and dry under vacuum at 80°C for 1-2 hours. Take off Saran wrap and expose the X-ray film at room temperature for 7 to 12 hours. Orient the film and dried gel with radioactive ink or needle punches or by piercing the film with a needle at three defined labeled points on the dried gel.

1.4. Reamplification of bands:

1. Orient the film exposure and the dried gel with radioactive ink (some ^{32}P + blue juice) previous to exposure.
2. Remove the Saran wrap, align the autoradiogram with the gel. Locate the bands of interest and cut through the film with clean scalpel blades.
3. Rehydrate the dried gel slice in 100 µl 1x TE at 80 ˚C for 10 min, spin briefly.
4. Transfer the supernatant to clean tube, precipitate with EtOH.
5. Resuspend the pellet in up to 100 µl 1 x TE.
6. Mix the following components of the PCR reaction: PCR Buffer, 1x; MgCl2, 2.5 mM; dNTPs, 250 µM; XSP6, 1 µM; BT7, 1 µM; Taq DNA Pol, 1.5 U. Total reaction volume 40 µl. Use 5 µl of the extracted DNA for the PCR.
7. Run the following thermocycler profile:

94°C, 2 min	initial denaturation

94°C, 45 s 40°C, 1 min 72°C, 1 min	}	5 cycles
94°C, 1 min 62°C, 1 min 72°C, 1 min/kb	}	35 cycles
72°C, 15 min		final extension

8. Check on 1.5-2 % agarose gel. Purify on low melting agarose for sequencing, clone if the band is not homogeneous.

2. ***Reverse Transcription***

1. Mix: 0.1-2 µg of total RNA; 2.5 µM of reverse primer (for ex., oligo(dT)). Final volume 18 µl. Heat for 5 min at 75 ˚C, cool down to 0 ˚C.
2. Add the following components: RT Buffer, 1x; DTT, 10 µM; dNTPs, 625 µM each; RNAsin 40 U; MMLV-RTase, 400 U. Total reaction volume: 40 µl.
3. Incubate for 1 hr at 37 ˚C, then heat for 10 min at 75 ˚C.
4. Remove excess dNTPs and primers by EtOH precipitation or commercially available kits.

3. ***Cloning of new members of the rat semaphorin family and Semaphorin-Specific Enriched Differential Display***

1) Total RNA is isolated from rat brain tissues using Ambion RNA isolation kit (RNAqueous, Cat. N°1912)
2) cDNA is synthetized from 1 µg of total RNA according to the Reverse Transcription protocol (§ II.2). Add water to a final volume of 100µl.

3.1. Semaphorin-specific RT-PCR with two degenerate primers

This protocol is designed to clone the new members of the semaphorin gene family using RT-PCR with two semaphorin-specific degenerate primers. PCR conditions are as previously described (Puschel et al., 1995).

1. Primers: forward: DDK; reverse: DPY or DPY/BamHI (see Table 2 for sequences).
2. Perform the PCR reaction with 10 or 20 µl of RT product in 100 µl final volume with the following reagents: PCR buffer, 1x (20 mM Tris.HCl pH 8.4; 50 mM KCl); $MgCl_2$, 3 mM; dNTPs, 0.25 mM, both primers, 1 µM; Taq Polymerase, 5 U
3. PCR program:

1x	denature template at 94°C for 4 min
5x	denaturation at 94°C for 1 min annealing at 60°C for 1.5 min elongation at 72°C for 1.5 min
5x	denaturation at 94°C for 1 min annealing at 55°C for 1.5 min elongation at 72°C for 1.5 min
5x	denaturation at 94°C for 1 min annealing at 50°C for 1.5 min elongation at 72°C for 1.5 min
5x	denaturation at 94°C for 1 min annealing at 45°C for 1.5 min elongation at 72°C for 1.5 min
20x	denaturation at 94°C for 1 min annealing at 55°C for 1.5 min elongation at 72°C for 1.5 min
1x	use a prolonged elongation time up to 10 min at 72°C

4. Analyze the samples on 2% agarose gel (estimated length of fragment: ≈900 bp).

For cloning new members of the semaphorin family a blunt-end ligation of the fragment into the appropriate vector (e.g. pBluescript) is performed. A Semaphorin-Specific Enriched Differential Display is performed as below.

3.2. Enriched Differential Display

PCR conditions and program like in the first reamplification after Differential Display (§II.1.4)

1. Primers: arbitrary or semaphorin-specific 25-mer primers (Table 2).
2. Perform the PCR reaction with 2 µl of the first PCR product in 100 µl final volume with the following reagents: PCR buffer, 1x (20 mM Tris.HCl pH 8.4; 50 mM KCl); $MgCl_2$, 2.5 mM; dNTPs, 0.25 mM, both primers, 1 µM; Taq Polymerase, 5 U
3. PCR program:

1x	denature template at 94°C for 2 min
5x	denaturation at 94°C for 45 sec annealing at 40°C for 1 min elongation at 72°C for 1 min
35x	denaturation at 94°C for 1 min annealing at 62°C for 1 min elongation at 72°C for 1 min
1x	use a prolonged elongation time up to 15 min at 72°C

4. *Riboprobe synthesis for non-radioactive in situ hybridization*

Labeled RNA probes are synthesized by in vitro transcription of DNA, cloned downstream of T3, T7 or SP6 RNA polymerases promoters, using digoxigenin- or fluorescein-labeled uridine-triphosphate as substrate. Alternatively, the probes can be generated from PCR template, containing the same promoters. The probes are detected, after hybridization to target nucleic acids, by enzyme-linked immunoassay using an antibody-conjugate (alkaline phosphatase or horseradish peroxidase). The product is visualized by enzyme-catalyzed chromogenic, fluorescent or chemiluminescent reaction.

1. Linearize 5 µg of insert-containing plasmid in 50 µl total volume reaction with 20U of corresponding enzymes and reaction buffers (see REMARK 1), for sense and antisense probes respectively. Incubate for 4 hrs. Check linearization efficiency on agarose gel.
2. Purify the template DNA on preparative agarose gel and purify the isolated band with an available Gel Extraction method (for ex., JETquick Gel Extraction Spin kit). Check the purified DNA on analytical agarose gel with DNA weight marker.
3. Label the RNA in a following reaction (if using the Kit for the first time, include controls; see REMARKS 2 & 3): transcription buffer, 1x; NTP labeling mix, 2 µl; DNA, ≈1 µg; RNA polymerase, 40U; RNase inhibitor (optional), 20U. Total reaction volume 20 µl.
4. Incubate for 2 hrs at 37°C.
5. DNA template removal (optional): Add 2-20U DNase I, RNase free, and buffer if needed, incubate for 15-30 min at 37°C. Add on ice 2 µl EDTA, 0.2 mol/l; pH 8.0 to stop the reaction.

Optional: Reduction of probe length (if needed, see remark 4): (adapted from (Angerer and Angerer, 1981; Brahic and Haase, 1978)):

Add ddH2O up to 50 µl and 50 µl of 0.2M carbonate buffer, pH 10.2.

Incubate for the appropriate time at 60°C. Hydrolysis time is given by the following relationship (Cox et al., 1984):

$$t = (lo-lf) / (kLoLf)$$

where

t = time in minutes
Lo = initial length in kilobases
Lf = desired length in kilobases
k = approximately 0.11 strand scissions/kilobases/minute.

Neutralize by adding 3 µl 3M NaOAc, pH 6.0, and 5 µl 10% (volume/volume) glacial acetic acid. Precipitate with 2 Vol Ethanol (see REMARK 5). Continue with 7).

6. Precipitate the labeled RNA with 1/5 Vol NH_4OAc, 10 M, and 3 Vol prechilled ethanol (see REMARK 5). Mix well. Leave for at least 30 min at –70°C or 2 h at –20°C. Centrifuge for 30 min at 4°C, wash the pellet with 80%, then with 100% Ethanol, dry with care.
7. Resuspend the probe in 100 µl ddH_2O.
8. Check the integrity of the probe on agarose gel (fluorescein-labeled RNA can be visualized directly w/o EtBr staining by UV transillumination). A better way is to check the probes on 2 to 2.5% agarose gel in MOPS buffer, containing 6% formaldehyde (Bruskin et al., 1982).
   The probes are stable for at least one year and can be stored in ddH_2O, hybridization solution, TE buffer or TE containing 0.1% SDS at –20°C.
9. Estimate the yield in a spot test with a DIG-labeled control (see separate protocol).

4.1. Remarks

1. It is of advantage to use the restriction enzymes creating 5'-overhangs. One also can prefer to make an additional cut in the middle of the vector in order to achieve better separation of DNA template for in vitro transcription from the rest of the plasmid on agarose gel.
2. If desired, the kinetics of the labeling reaction can be followed and the amount of newly synthesized RNA determined by the addition of a radioactively labeled tracer NTP (30 nm {α-^{32}P}-UTP) and TCA or ethanol precipitation.
3. In this reaction up to 20 µg of RNA per µg linearized template DNA are synthesized (approx. 100-fold transcription of insert). Scaling up the reaction volume and components, while keeping the amount of template DNA constant can improve the yield of DIG-labeled RNA.
4. Probes up to 1200 bp in length penetrate the tissue effectively.
5. The alternative to precipitation would be the purification with Boehringer Quick Spin columns.

5. *Estimating the yield of DIG-labeled riboprobe in a spot test with a DIG-labeled control*

The dilutions of newly prepared DIG-labeled riboprobe are spotted on a positive nylon membrane together with the corresponding dilutions of DIG-labeled control RNA of known concentration in order to appreciate the efficiency of the labeling reaction.

5.1. Membrane preparation

1) Make a predilution of the DIG-labeled Control RNA (Boehringer, Cat.No.1585746) in H_2O to a final concentration of 20 ng/µl.
2) Make serial dilutions of the prediluted control, according to the following dilution scheme. Mix thoroughly between dilution steps.

DIG-labeled Control RNA, Starting Concentration	Total dilution	Final concentration (dilution name)
20 ng/µl	1:20	1 ng/µl (A)
1 ng/µl (dilution A)	1:200	100 pg/µl (B)
100 pg/µl (dilution B)	1:2,000	10 pg/µl (C)
10 pg/µl (dilution C)	1:20,000	1 pg/µl (D)
1 pg/µl (dilution D)	1:200,000	0.1 pg/µl (E)
0.1 pg/µl (dilution E)	1:2,000,000	0.01 pg/µl (F)

   Highly diluted solutions of RNA in H_2O are not very stable. Spots must be made immediately after preparing the dilutions. Alternatively the RNA can be diluted in RNA dilution buffer (DMPC-treated water, 20xSSC and formaldehyde, mixed in a volume ratio of 5+3+2) for greater stability.
3) Estimate the expected yield of *in vitro* transcription reaction[1]. Predilute an aliquot of the newly synthesized experimental RNA probe to an expected final concentration of approx. 20 ng/µl.
4) Make serial dilutions of the prediluted experimental probe[2], according to the scheme (table).
5) Spot 1 µl of the diluted controls (C-F) on a piece of nylon membrane.
6) In a second row, spot 1 µl of the corresponding dilutions of the experimental probe.
7) Fix the nucleic acids to the membrane by cross-linking with UV-light or by baking for 30 min at 120°C (Boehringer Mannheim Nylon Membrane). Proceed with standard detection procedure.

[1] Good estimation for the *in vitro* transcription reaction is 1:40 template:product ratio (in fact it often gives 1:100 and even higher ratios). It means that if 1 µg of 3000 bp plasmid with 300 bp insert was used for probe preparation, the actual template was at 100 ng in the reaction, and the reaction will result in 4 µg of riboprobe.

[2] It is actually more useful to titrate the control RNA with lower dilution factor (for example, 2x) to have a more representative scale as a marker to compare with experimental dilutions.

5.2. Detection procedure for a 100 cm^2 membrane:

1) Rinse the membrane briefly (1-5 min) in washing buffer (100 mM maleic acid, 150 mM NaCl; pH 7.5; 0.3% (v/v) Tween® 20).
2) Incubate for 30 min in 100ml 1x Blocking solution (1% Blocking reagent (Boehringer) solution in 100 mM maleic acid, 150 mM NaCl; pH 7.5).
3) Dilute anti-DIG-AP 1 to 5000 in 1x Blocking solution. Mix gently by inversion. This solution is stable for about 12 h at 4°C.
4) Incubate the membrane for 30 min in 20 ml antibody solution.
5) Wash 2 times for 15 min with 50 ml washing buffer.
6) Equilibrate for 2-5 min in 20 ml detection buffer (100 mM Tris-HCl, pH 9.5; 100 mM NaCl).

7) Add 45 µl NBT solution and 35 µl BCIP solution in 10 ml of detection buffer. This color substrate must be prepared freshly.
   or
   Dilute CSPD® (vial 5) 1:100 in the detection buffer (this solution can be reused 1-2 times within a month. Store in the dark at 4°C).

In case of chemiluminescent detection continue with step 11.

8) Pour off the detection buffer and add the color substrate solution. Allow the color development to occur in the dark. The color precipitate starts to form within a few minutes and continues for approx. 16 h. Do not shake while the color is developing.
9) When the spots appear in sufficient intensity, stop the reaction by washing the membrane with TE buffer or sterile H_2O for 5 min.
10) Compare spot intensities of the control and experimental dilutions to estimate the concentration of the experimental probe.
11) Place the membrane between two sheets of Saran wrap. Gently lift the top sheet and, with a sterile pipette, add approximately 0.5-1 ml (per 100 cm^2) of the chemiluminescent substrate on top of the membrane, scattering the drops over the surface of the membrane. Lower the top sheet of plastic. With a damp lab tissue gently wipe the top sheet to remove any bubbles present under the sheet and to create a liquid seal around the membrane. Incubate filter for 5 min.
12) Let excess liquid drip off and blot the membrane briefly (DNA side up) on Whatman 3MM paper. Do not let the membrane dry completely!
13) Envelop the membrane into the sheet of Saran wrap and incubate for 5-15 min at 37°C to enhance the luminescent reaction.
14) Expose for 15-20 min at room temperature to X-ray or Polaroid b/w film. Multiple exposures can be taken (luminescence continues for at least 24 h and signal intensity increases during the first hours).

6. *In situ hybridization*

6.1. Brain isolation and cutting:

1) Remove the brains.
2) Freeze rapidly in cold isopentane (methylbutane) on dry ice. After 5-10 min transfer the brains to clean 50 ml Falcon tube.
3) Store dry at –70 to -80°C. Continue with 4).

alternatively (Tanimura et al., 1998)

1') Remove the brains.
2') Keep in ice-cold 4% paraformaldehyde for 2-4 days.
3') Freeze in dry ice-cold hexane (5-10 min). Store dry at -80°C.
4) Cut the brains on sections of 25 µm of desired orientation.
5) Dry out the slides with sections for at least 1 hr at RT and then store in tightly closed plastic boxes (Parafilm) with Silicagel at –20°C. For *in situ* studies remove the slides from the boxes at –20°C (be aware of evaporation) or take the boxes out of the freezer and keep them for 1 hr at RT prior to opening.

alternatively

6) Fix cut sections in freshly prepared 4% paraformaldehyde, ice-cold, for 10 min, wash in 1xPBS for 15 min, and store in 70% EtOH at 4°C.

It may be important to proceed to the next steps of protocol immediately after cutting to keep rare transcripts intact in cryosections.

6.2. Tissue treatment and hybridization (adapted from (Braissant and Wahli, 1998)):

6.2.1. Post -fixation:

1) Incubate the slides for 10 min in 4% paraformaldehyde.
2) Incubate twice for 15 min in 1xPBS with 0.1% active DEPC.
3) Wash for 10-15 min in 5xSSC.

6.2.2. Pre-hybridization:

Both pre- and hybridization are carried out in plastic boxes saturated with a 5xSSC / 50% formamide solution to avoid evaporation.

1) Add 250 µl of hybridization buffer (50% formamide, 5XSSC) with 40µg/mL salmon sperm DNA (GibcoBRL, Cat.No. 15632-011) per section.

2) Grease covers to hybridization boxes, close firmly with parafilm (drying of sections during this step can lead to substantial background!).
3) Incubate for 1-2hrs at 55-61°C (the same temperature as for hybridization) in hybridization oven.

6.2.3. Hybridization:

1) Prepare riboprobe working solutions at conc. 40-400 ng/ml. Heat probe mix to 80°C for 5 min.
2) Add 20µl of probe per section, cover with a square of parafilm. Again close the box firmly with parafilm and put in oven at 55-61°C (depending on the particular probe) for O/N (4-40 hrs depending on transcript abundance).

6.2.4. Washings and detection:

1) 30min in 2XSSC at RT.
2) 1hr in 2XSSC at 65°C.
3) 1hr in 0.1XSSC at 65°C.
4) If needed (should be tested for each probe):
   20min RNase treatment, 30µg/ml, at 30°C
   wash 2x for 15min in 2XSSC at RT.
5) Equilibrate for 5min in Buffer 1 (Tris-HCl, 100 mM; NaCl, 150mM; pH 7.5).
6) Incubate for 2hrs at RT with 150µl/section of Buffer 2 (Buffer 1 + 0.5% Boehringer blocking reagent) with 5000x dilution of antibodies (DIG or fluorescein).
7) Wash 2x for 15min in Buffer 1.
8) Equilibrate for 5 min in Buffer 3 (Tris-HCl, 100mM; NaCl, 100mM; $MgCl_2$ 50mM; pH 9.5).
9) Incubate with Buffer 3 along with 4.5µl/mL NBT, 3.5µl/mL BCIP (150µl/slide) in dark overnight up to several days at RT.
10) Wash for 15min in 1XTE.
11) Wash for 1hr (up to O/N) in 95%EtOH (with gentle agitation).
12) Rinse for 15min in ddH_2O.
13) Let air dry and use xylene (xylol) and Eukitt resin to place coverslips.

6.2.5. Useful tips

a) If nuclear staining is required, slides can be counterstained with DAPE.
b) Following steps must be normalized for each probe:
   hybridization T° (55-62°C)
   hybridization time (4-40 hrs)
   probe concentration (40-1000 ng/ml)
   coloration time (1 hr – several days)
   RNase treatment (20-40 µg/ml, RT – 37°C).
c) The following controls are useful: no probe, RNase treatment prior to hybridization (after first DEPC incubation: 5x SSC, 5 min; RNase 50 µg/ml, 30 min at 37°C; continue with second DEPC incubation), probes to different parts of the mRNA.

7. *Quantitative RT-PCR*

This protocol has been developed to perform quantitative RT-PCR on iCycler (Biorad). It is based on Perkin-Elmer recommendations and protocols. The quantification of a target is calculated relative to an internal standard (28s rRNA) to account for variability in the initial concentration and quality of the total RNA and in the conversion efficiency of the reverse transcription reaction. The measure of SYBR Green fluorescence at the threshold cycle is used for quantification of PCR products.

All cDNAs are prepared from 100 ng of total RNA, according to the Reverse Transcription protocol (§II.2).

Terms used:

Standard: cDNA prepared from total brain RNA (actually this can be any RNA of good quality, containing the sequence of interest). It is used in the PCR at different dilutions, allowing for regression curve construction.
NTC: no template control. One can use water, or better cDNA prepared in the same conditions as standard, but without the RTase.
Unknown: cDNA prepared from brain regions of interest at different experimental conditions.

Protocol:

1) Design primers using PrimerExpress software according to program instructions.

2) Optimize primer concentrations: run PCR on any cDNA template with all combinations of forward and reverse primers at 50, 300 and 900 nM, respectively. Choose the concentration giving the lowest threshold cycle and highest end-product concentration.
3) Prepare cDNAs.
4) Prepare the plate configuration: e.g. use five standard dilutions, each 5x, starting from 4x dilution of initial RT. All standards are run in triplicate. The unknown cDNA are diluted 10x, and are run in quintuplicate. These parameters can be adapted depending on particular experimental needs.
   One half of the plate is normally devoted to 28s rRNA, the second half to the primers specific for the gene of interest. However, if rRNA values remain stable when the experiment is repeated, it may be not necessary to include these reactions in each plate.
5) Prepare the dilutions of the standard.
6) Prepare 10x dilutions of NTC and of all unknown cDNAs.
7) Prepare MasterMix 2x, containing: PCR buffer, 2x; $MgCl_2$, 6 mM; dNTPs each, 400µM; HotStarTaq, 2U/25 µl. The HotStarTaq Polymerase as well as PCR buffer and $MgCl_2$ solutions are from Qiagen (1000U, Cat.No.203205). The mix can be stored at 4°C.
8) Prepare standard pool, containing: 2x MasterMix, 1x; SYBR 5x, 0.2x (SYBR Green I, 10000x in DMSO, Molecular Probes, Cat.No.S-7563); forward and reverse primers, 50, 300 or 900 nM respectively. Final volume 45 µl.
9) Pipette 45 µl of mix into the plate wells, add 5 µl of corresponding cDNAs.
10) Adjust the mask (normally to be performed once during the iCycler installation) and calibrate the machine.
11) Program the iCycler. The quantities for the standards are chosen arbitrarily (e.g. Start with 100 ng, and then continue according to dilution factor).
12) Run the following thermocycler profile:

Times and Temperatures		
HotStarTaq Activation	Each of 40 cycles	
	Melt	Anneal / Extend
HOLD	CYCLE	
10 min 95°C	15 sec 95°C	30-60 sec 60°C

13) Save and analyze the data. The analysis includes the standard curve calculation and the determination of unknowns. The following parameters may be modified to obtain the best correlation coefficient:
   a) Baseline cycles
   b) Threshold
   c) Number of data points used in the analysis
   d) Wells included in the analysis.
14) Once the optimal values are obtained, transfer them to Excel to continue the calculations (normalization relative to the internal standard, statistics, and graphical representation).

8. *Ligation in Low Melting Agarose*

If blunt-end ligation with PCR fragments is performed, it is necessary first to treat them with T4 DNA Polymerase in order to remove the adenine, added during the PCR reaction. It is also advised to phosphorylate the PCR fragments further with T4 polynucleotide kinase with parallel dephosphorylation of the linearized vector with calf intestine phosphatase to increase the efficiency of ligation.

1) Separate the DNA restriction fragments on a "low melting" agarose gel with 1xTAE buffer and 0.5 µg/ml ethidium bromide both in the gel and in the running buffer. Load ca 1µg of total DNA per lane (for a plasmid of 3 kb such as an empty cloning vector, e.g. pUC, Bluescript) or proportionally more or less DNA for bigger or smaller DNAs. If the concentration of the PCR fragments is not precisely known, it can be estimated by comparison to vector bands on a preparative gel picture.

   The important parameters in a ligation are:
   the equimolarity of each DNA fragment
   a concentration < 0.5 fmol/µl of each fragment
   Assuming the presence of 1µg of a fragment of 3 kb (e.g. corresponding to 0.5 pmol of linearized cloning vector) in an excised band of 100 µl volume, the contribution of 5 µl of melted agarose of this fragment into 50 µl end ligation volume leads to a final concentration of 0.5 fmol/µl, so much as required; higher concentrations lead to the formation of multimers, too low concentrations preferentially lead to intramolecular reaction (e.g. the vector closing itself).

2) Take a picture of the gel. Cut out the bands with a clean scalpel blade with the aid of a UV hand lamp or on the UV transilluminator (don't expose too long to UV light) and transfer them into sterile ED tubes. Adjust the volume to 100 µl if needed.
3) Melt the excised DNA bands in the heating block at 65 ˚C (for higher percentage gels, e.g. 2%, it may be necessary to increase the temperature by some degrees to completely melt the agarose), mix well with vortexer, quick spin and keep in the heating block. Put the tubes with the prepipetted ligation buffer (5x T4 DNA Ligase Buffer, GibcoBRL) and ddH_2O in the heating block at 37 ˚C.
4) Prewarm each pipette tip in the water-containing ED tube at 65 ˚C aspirating up and down, pipette DNA fragments where required into the tubes at 37 ˚C. The melted agarose will remain liquid until kept at this temperature.
5) While keeping the tubes at 37 ˚C, add 1-2 µl of T4 DNA Ligase (Gibco BRL, Cat.No.15224-017) at 0.2 U/µl or 1 µl of ligase at 1 U/µl (keep the enzyme in a cooling block (-20˚C)). Mix well, quick spin, incubate at 4 ˚C or RT for few hours to O/N.

   General rules:
   Ligations with overhangs: 0-4 ˚C; 0.4 U ligase/ tube; 3-4 hours is enough. Ligations with only blunt ends: temperature irrelevant for "hybridization"; 0.4 - 1 U ligase/tube; 3-4 hours at least. Ligations with mixed ends: temperature gradient (put the tubes on ice in beaker and let them until the ice is completely melted); 0.4- 1 U ligase/tube; O/N. If more than 5 fragments must be ligated simultaneously, higher concentration of ligase is required (1 U/tube).
6) For transformation of bacteria: melt ligation again at 65 ˚C before pipetting 10 µl to an aliquot of $CaCl_2$ competent bacteria on ice (for transformation prot. see §II.9).

9. ***Transformation of bacteria:***

1) For each DNA to be transfected into the bacteria prepare a ED-tube on ice. Prepare an additional ED-tube for the transformation control (any vector, at 0.04 ng/µl).
2) Resuspend well the competent bacteria using a micropipette, distribute 100 µl (≈ 10^8 bacteria) in the precooled, appropriately labeled ED-tubes, keep tubes on ice.
3) Add 10 µl of the corresponding plasmid DNA (ca 1 ng) or one third of the ligation mix (liquefy at 65°C if low-melting agar) to the bacteria, mix well with the pipette (never use vortexer).
4) Incubate on ice for 5 min.
5) Put the tubes for 3 min into a water bath at 37˚C ; let stand for ca 5 min on ice.
6) Pipette ca 1 ml of L-Broth medium prewarmed to 45˚C to the bacteria tubes. Close the tube and mix well (invert , don't vortex).
7) Incubate for 5 min in the water bath at 37 ˚C (mix occasionally). During this time bacteria will recover and start synthesizing resistance genes. There should be no more than one duplication.
8) Spin down the bacteria in a ED-centrifuge (ca 30 seconds is necessary), aspirate all supernatant but ca 100 µl.
9) Resuspend the bacteria well. Plate them onto an appropriate agar plate.
10) Put the dishes turned upside down into a 37 ˚C incubator and incubate overnight.

10. ***SCOP (Single Colony Prep)-Assay***

The aim of this protocol is to quickly analyze the size of plasmid DNA from a large number of transformed bacterial colonies or streaks. This allows to identify the colonies carrying the recombinant plasmid DNA of the desired length. The alternative method is analytical PCR on colonies with the primers flanking the insert.

1) Gently aspirate an entire colony or 0.5 cm length of a streak with 20 µl pipette and resuspend in the LETR (lysozyme, 2 mg/ml; EDTA pH 8, 100 mM; Tris pH 8, 50 mM; RNaseA, 0.1 mg/ml). Incubate for 20-30 min at RT.
2) Add one drop of phenol to each tube, mix, centrifuge for about 3 min.
3) Take 5 µl supernatant and load onto gel together with comparative markers.

11. ***Minipreparation of DNA***

This protocol allows to purify plasmid DNA from transformed small bacterial cultures.

11.1. Growth:

1) Inoculate with one bacterial colony or streak 5 ml LB medium supplemented with 100µg ampicillin (or other antibiotic).
2) Grow the culture at 37°C in the shaker for 18-24 h (up to 36 h).

11.2. Lysis & extraction:

1) Centrifuge the bacteria, resuspend the pellet in 400 µl of ice cold LETR (lysozyme, 2 mg/ml; EDTA pH 8, 100 mM; Tris pH 8, 50 mM; DNase-free RNaseA, 0.1 mg/ml). Mix well. Transfer into Eppendorf tube, Incubate for 20-30 min at RT.
2) Add 1 drop of 10% Triton X-100, mix gently. Incubate for further 5 min at RT (optional). Centrifuge for 10-15 min at 13 kRPM.
3) Remove the pellet.
4) Extract 1x with phenol, 1x with phenol:chloroform 1:1, 1x with chloroform (400 µl each, transfer the aqueous phase onto a fresh tube each time)
5) Adjust the volume to 400 µl with water, add 40 µl 3M NaCl and 240 µl isopropanol. Incubate for 10 min at RT.
6) Centrifuge for 10 min at RT at 13 kRPM.
7) Discard the supernatant, wash the pellet 1x 80% EtOH and 1x 100% EtOH. Let dry, resuspend the DNA in 100 µl TE.

12. *Plasmid Preparation*

This protocol allows to purify plasmid DNA from transformed bacterial cultures of 100 ml. The amount of DNA recovered should be 500-1000µg.

12.1. Growth:

1) Inoculate with one big bacterial colony or streak 100 ml LB medium supplemented with 10 mg ampicillin (or other antibiotic).
2) Grow the culture at 37°C in the shaker for 18-24 h (better 36-48 h). Optional: to improve the yield let stand the grown culture at 4°C for 24-72 hr.

12.2. Lysis:

1) Centrifuge the bacteria in a 100 ml tube at 3000-4000 RPM for 15-20 min at 4°C. The pellet can be stored at -20°C in Falcon 50 ml tubes.
2) Resuspend the pellet in 6-7 ml of ice cold LETR (if frozen pellet is used, do not allow to thaw prior to LETR addition): lysozyme, 2 mg/ml; EDTA pH 8, 100 mM; Tris pH 8, 50 mM; DNase free RNaseA, 0.1 mg/ml. Mix well. Transfer into an Oak Ridge tube. Incubate for ~ 30 min at RT (the suspension should get slightly viscous).
3) Add 100 µl 10% Triton X-100, mix (2-3 strong strokes, rotate). The suspension should get very viscous. Incubate for further 10-30 min.
4) Centrifuge at 10'000-15'000 RPM for 30-40 min at 4°C. Transfer the clear supernatant to a fresh corex tube or another suitable tube for organic extractions.

12.3. Purification:

1) Extract 1x1 vol. phenol, 1x1 vol. phenol/chloroform and 1x1 vol. chloroform.
2) Adjust the volume to 7 ml. Add 0.7 ml 3M NaCl (3M NaOAc can also be used), add 4.2 ml isopropanol. Mix well, incubate for ~10 min at RT and spin at 10'000 RPM for 15-20 min.
3) Discard the supernatant and rinse the pellet carefully with 80% ethanol and with 100% ethanol. Let dry.
4) Resuspend the pellet in 0.5-1 ml TE.

Note: For bigger cultures, scale up the volume of lysis, extractions and precipitation.

Table 2. Primers used in the present study

Name of primer	Sequence
Oligo-dT Primers	
DLPGG	5'-C ACT ATA GGG AAG CTT TTT TTT TTT GG-3'
DLPGA	5'-C ACT ATA GGG AAG CTT TTT TTT TTT GA-3'
DLPGT	5'-C ACT ATA GGG AAG CTT TTT TTT TTT GT-3'
DLPGC	5'-C ACT ATA GGG AAG CTT TTT TTT TTT GC-3'
DLPAG	5'-C ACT ATA GGG AAG CTT TTT TTT TTT AG-3'
DLPAA	5'-C ACT ATA GGG AAG CTT TTT TTT TTT AA-3'
DLPAT	5'-C ACT ATA GGG AAG CTT TTT TTT TTT AT-3'
DLPAC	5'-C ACT ATA GGG AAG CTT TTT TTT TTT AC-3'
DLPCG	5'-C ACT ATA GGG AAG CTT TTT TTT TTT CG-3'
DLPCA	5'-C ACT ATA GGG AAG CTT TTT TTT TTT CA-3'
DLPCT	5'-C ACT ATA GGG AAG CTT TTT TTT TTT CT-3'

DLPCC	5'-C ACT ATA GGG AAG CTT TTT TTT TTT CC-3'
Oligo-dT Primer Sets	
DLP1	5'-C ACT ATA GGG AAG CTT TTT TTT TTT VG-3'
DLP2	5'-C ACT ATA GGG AAG CTT TTT TTT TTT VC-3'
DLP3	5'-C ACT ATA GGG AAG CTT TTT TTT TTT VA-3'
DLP4	5'-C ACT ATA GGG AAG CTT TTT TTT TTT VT-3'
DLPG	5'-C ACT ATA GGG AAG CTT TTT TTT TTT GN-3'
DLPA	5'-C ACT ATA GGG AAG CTT TTT TTT TTT AN-3'
DLPC	5'-C ACT ATA GGG AAG CTT TTT TTT TTT CN-3'
Arbitrary Primers (last 10 nucleotides are arbitrary and vary in each primer)	
XAp1	5'-ACA CTA TAG CTC GAG AGG TGA CCG T-3'
XAp2	5'-ACA CTA TAG CTC GAG TGG ATT GGT C-3'
XAp3	5'-ACA CTA TAG CTC GAG CTG ATC CAT G-3'
XAp4	5'-ACA CTA TAG CTC GAG TTT TGG CTC C-3'
XAp5	5'-ACA CTA TAG CTC GAG GGA ACC AAT C-3'
XAp6	5'-ACA CTA TAG CTC GAG AAA CTC CGT C-3'
XAp7	5'-ACA CTA TAG CTC GAG TCG ATA CAG G-3'
XAp8	5'-ACA CTA TAG CTC GAG TGG TAA AGG G-3'
XAp9	5'-ACA CTA TAG CTC GAG TCG GTC ATA G-3'
XAp10	5'-ACA CTA TAG CTC GAG GGT ACT AAG G-3'
XAp11	5'-ACA CTA TAG CTC GAG TAC CTA AGC G-3'
XAp12	5'-ACA CTA TAG CTC GAG CTG CTT GAT G-3'
XAp13	5'-ACA CTA TAG CTC GAG GTT TTC GCA G-3'
XAp14	5'-ACA CTA TAG CTC GAG GAT CAA GTC C-3'
XAp15	5'-ACA CTA TAG CTC GAG GAT CCA GTA C-3'
XAp16	5'-ACA CTA TAG CTC GAG GAT CAC GTA C-3'
XAp17	5'-ACA CTA TAG CTC GAG GAT CTG ACA C-3'
XAp18	5'-ACA CTA TAG CTC GAG GAT CTC AGA C-3'
XAp19	5'-ACA CTA TAG CTC GAG GAT CAT AGC C-3'
XAp20	5'-ACA CTA TAG CTC GAG GAT CAA TCG C-3'
Universal reamplification primers	
XSP6	5'-GCT CTA GAT TTA GGT GAC ACT ATA GCT CG-3'
BT7	5'-GCG GAT CCT AAT ACG ACT CAC TAT AGG GAA-3'
Semaphorin-specific degenerate primers	
DDK forward primer (Luo et al., 1995)	
AA sequence Nucleotide sequence	DDKYIFFF 5'-GAY GAY AAR ATH TAY TTY TTY TT-3'
DPY reverse primer (Luo et al., 1995)	
AA sequence Nucleotide sequence	DPYCAWD 5'-TCC CAN GCR CAR TAN GGR TC-3'
DPY/BamHI (DPY reverse primer + site BamHI)	
Primer + *BamHI* site	5'-CG *GGATCC* CAN GCR CAR TAN-3'
Semaphorin-specific Display primers	
Forward	
XColl1	5'-ACA CTA TAG CTC GAG ACT TCT TCT T-3'
XColl2	5'-ACA CTA TAG CTC GAG TCC TGA AGG C-3'
Reverse	
HColl3	5'-CAC TAT AGG GAA GCT TGT GGT GAA GA-3'
HColl4	5'-CAC TAT AGG GAA GCT TAT CTC CTC CA-3'

Primers used for differential display, semaphorin-specific RT-PCR and enriched differential display studies. For particular use refer to METHODS section.

III. RESULTS

1. *Modification of primer design in differential display (Annex I)*

Numerous methods are currently available allowing for simultaneous detection of gene expression changes in several samples. The method of differential display (DD) (Liang and Pardee, 1992) has gained a large audience in past years. Its inherent simplicity makes it preferable to older methods such as subtractive hybridization. It has also a big advantage compared to more recently developed microarrays, because it allows for detection of differential expression of virtually all mRNAs present in the cells independently of their previous identification and cloning. Nevertheless, false positives generated by the PCR step as well as laborious identification of differentially expressed genes remain a major drawback of the method. Therefore we have decided to introduce two modifications into the original DD protocol.

First, as proposed previously, we used longer primers instead of the original 10-mers. This modification integrates the principles of DD with those of arbitrarily primed PCR (Welsh et al., 1992). Accordingly, PCR is performed in two steps. Few initial low-stringency cycles are followed by several high stringency cycles favoring more specific hybridization in the PCR.

Second, we took advantage of increased primer length to introduce additional elements into the primer sequence aimed at facilitating reamplification, analysis and cloning of the cDNA fragments. The 5' terminus of the oligo(dT) primers and the arbitrary primers are extended with about one half of the T7 and SP6 RNA polymerase promoters, respectively. Universal re-amplification primers harboring the complete cognate promoter sequences will prime within these extended constant regions. Thus, the final amplicon will contain two functional RNA polymerase promoters allowing for direct generation of sense and antisense riboprobes for hybridization techniques such as Northern blot, *in situ* hybridization or ribonuclease protection analysis. This configuration allows us to vary the arbitrary and the oligo(dT) portion in the initial PCR primers while maintaining the same universal reamplification primer pair. In addition, the same particular re-amplification primer can be used for direct sequencing of all reamplified cDNAs.

Finally, four restriction sites flanking the promoter sequences are introduced into the final amplicon, thus providing increased flexibility for cloning purposes.

The performance of the modified primers is illustrated in the following paper (Brenz Verca et al., 1998) using FTO-2B rat hepatoma cells. The induction of the tyrosine aminotransferase gene expression by glucocorticoids is used as a positive control to check for the reliability of the new method.

2. *Changes in gene expression upon cocaine treatment*

In order to examine the changes in gene expression in the dopaminergic brain system upon acute cocaine treatment, we used the modified DD protocol described above (Section I, (Brenz Verca et al., 1998)). Rats received four subcutaneous injections of 30 mg/kg cocaine-HCl or saline every 2 hrs and were sacrificed 24 hrs after the last injection. The experiments were performed in two sets. First, the RNA from three brain regions, the nucleus accumbens (NAcc), the lateral striatum (LStr) and the hippocampus (Hipp) upon saline- and cocaine- treatment respectively were isolated and analyzed in parallel. This allowed us to compare not only drug-induced changes, but also basic differences between the regions. Then, a separate DD was performed on the ventral tegmental area (VTA) of saline versus cocaine-treated animals. Results, as well as general observations about the types of genes found in the screening, will be presented below.

2.1. Changes in gene expression in NAcc, LStr and Hipp

Twenty arbitrary primers were used in combination with twelve oligo(dT) primers. In order to reduce the complexity of the DD pattern, the oligo(dT) primers were combined into 3 sets of 4 primers, each set with the penultimate 3' nucleotide constant. Such sets contain the primers which are more compatible in terms of annealing temperature than sets with the last 3' nucleotide constant, normally used in the majority of DD protocols. The sets were called DLPG (DLP for dT lower primer), DLPA, and DLPC, for respectively, G, A or C as the penultimate 3' nucleotide.

All PCRs were performed in triplicate starting from independent reverse transcription (RT) reactions. The initial PCR amplifications were run always with two controls, by omitting either cDNA in the PCR reaction or the RTase in the RT step. In both control reactions no bands were visible (not shown), so the DD pattern was determined by the primers and the cDNA template, and not by autoannealing of the primers nor by the amplification of remaining genomic DNA or any other contaminating templates. Patterns of expression were compared for RNAs isolated from the NAcc, the LStr, and the Hipp of saline- or cocaine-treated rats. The fragments found in DD did not exceed 600 bp, and those smaller than 100 bp were not proceeded for further analysis in order to exclude all possible degradation products from the screening results.

2.1.1. Two sets of signals found in differential display

The overall pattern of gene expression in the three brain regions investigated can be assigned to two distinct sets of signals (Figure 26):

1) The predominant pattern (about 95%) displayed gene expression at similar intensities in all three regions in saline-treated brains. These signals were often found at the same level on gels with several primer combinations. In this case each band, probably consisting mostly of house-keeping genes, remained unchanged upon cocaine treatment, except for one signal.
2) The second type of pattern displayed a different basal level of expression for three or at least two of the investigated regions, and there about half of the candidates displayed changes in expression upon cocaine treatment.

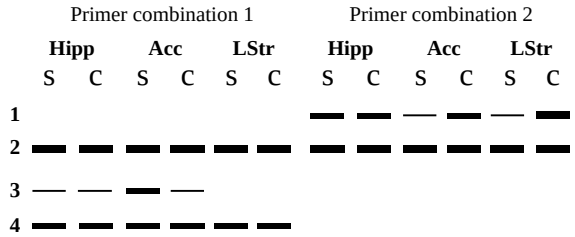


Figure 26. Examples of differential display patterns found by comparing the three regions upon cocaine treatment. Numbers at left are related to corresponding bands on the gel. N 2 and 4 are the typical examples of the first set of signals (see text for explanations): their expression level is the same in all regions under basal conditions, and remains unchanged upon cocaine treatment. In addition, N2 is present with two different primer combinations. N 1 and 3 are examples of the second set of signals: their expression is detected by one particular primer combination, the basal expression level is different depending upon the region examined, and their expression changes upon cocaine. s: saline, c: cocaine.

This second set of signals, with a region-specific pattern, was found with only 12 out of the 20 arbitrary primers. Three particular arbitrary primers among these 12 were especially efficient in this respect, giving rise to one half of such signals. This means that each arbitrary primer has a different capacity to detect the variations in expression, both region- and drug-dependent. Eight out of the 20 arbitrary primers did not reveal any differences in expression between the various genes. Maybe for these 8 arbitrary primers the experimental conditions were sub-optimal or their structure less suitable for PCR in general, and thus they amplified only highly abundant messages, e.g. house-keeping genes present in all regions and hardly modified by drug treatment. It is also conceivable however that these 8 primers were highly efficient PCR primers, allowing for the DD reach the saturation point, when the detection of specific differences is impossible.
Whatever the explanation, it is necessary to vary the PCR parameters in order that all primers work efficiently.

2.1.2. Analysis of differentially expressed signals in the three brain regions

We analyzed in more detail 40 signals which display differential expression in the three brain regions at least in duplicate experiments. This analysis was performed prior to the sequencing of particular candidates, and was aimed at drawing general conclusions about the patterns of gene expression in the different regions prior and following the drug treatment. One of the questions we addressed was whether the pattern of gene expression may serve as a useful criterion for defining the morphological and functional identity of a particular brain region. If it were the case, the two regions of the striatum (NAcc and LStr) must possess higher similarities in gene expression pattern compared to an anatomically and functionally distinct region such as Hipp. We found some general pattern in gene expression among the different brain regions:

1. 19 signals were expressed in every region examined (Figure 27A), but at different levels depending upon the region. 20% of the signals were expressed only in the Hipp. Conversely, no signal was found exclusively in the NAcc.
2. The level of expression in the Hipp was different from the NAcc and the LStr in about 80% of the cases, whereas the level of expression in the last two regions was the same in about half of the cases (Figure 27B). In fact this observation confirms our presumption that the gene expression pattern may be a useful marker of a functional and neuroanatomical identity of a particular brain region.
3. The majority of signals were stronger in the Hipp (60%) and weaker in the NAcc (80%) than in one or both of the other regions (Figure 27C). The latter was especially striking compared to LStr. In all cases where differences between the NAcc and the LStr were observed, only once was it stronger in the NAcc.

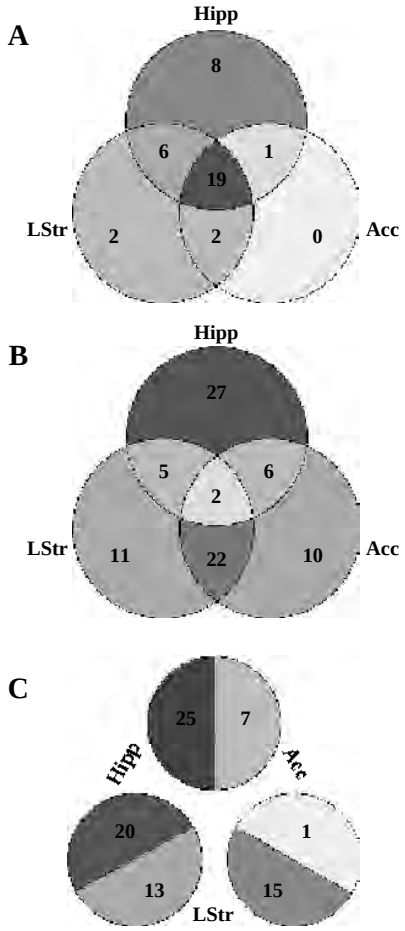


Figure 27. General features of region-specific gene expression pattern in DD. A. Expression of 40 sequences with different levels of expression in three brain regions. Each circle represents a particular brain region. Overlaps between the circles contain the sequences present in several regions at the same time. The abundance of signals in each group is represented by the intensity of the colors and numbers inside the sections. Note that the largest group of candidates is expressed in all three regions, and that the NAcc does not contain a specific signal. B. Distribution of candidates with similar level of expression between regions. Each circle represents a particular brain region. Overlaps between the circles show the number of candidates with similar level of expression between two or all three regions. Colors and numbers as in A. Note that the total number is not 40, because in this case not every sequence was analyzed, but every individual difference between regions. C. Distribution of signals regulated among two different regions. Each circle is divided into two hemispheres where the number inside the left half corresponds to the number of signals up-regulated in the left-side region compared to the right-side one. So, e.g. numbers in the upper circle indicate that 25 signals were up-regulated in the Hipp compared to the NAcc, and 7 signals were up-regulated in the NAcc compared to the Hipp. Note that the majority of signals is up-regulated in the Hipp and down-regulated in the NAcc compared to other regions. Colors and numbers as in A. Acc: nucleus accumbens.

Signals not modified by drug treatment are presented in Table 3. The majority of differences were found with DLPA set of oligo(dT) primers. The differential expression of 12 candidates was confirmed in triplicate DD experiments. Some of the candidates have been sequenced. Only one fragment (No11 in the Table 3) was homogeneous enough to be sequenced directly without cloning. Highest homology was found to the murine protein HP1-BP74. This protein is the mouse homologue of the Drosophila heterochromatinic protein 1 (Le Douarin et al., 1996). It interacts with TIF1, a mediator of ligand-inducible activation of the nuclear receptor. The NAcc-specific expression of this factor may reflect the region-specific control of transcription, exerted at the level of the structure of the chromatin template.

The remaining bands were cloned into pBSKS+, and at least ten monoclonal plasmid preparations were analyzed for each separate fragment. Restriction analysis was then performed to assess the level of sequence heterogeneity in each particular band. Normally this analysis resulted in defining several sequences present in one signal; however one, or rarely two sequences were predominant, and these particular clones were sequenced.

The analysis of sequences allowed also for evaluation of the possible redundancy of signals, a major drawback inherent to DD experiment. Sometimes the PCR product may be generated by only one of the primers from the oligo(dT)-arbitrary pair working on both sides of the amplicon. We suspected this to happen in our DD, because some of the bands appeared at the same level on the gel and showed the same pattern of expression independently of the arbitrary primers used. However, in all fragments analyzed upon sequencing we found both primers present.

Five sequences (No12,clone44; 14,clone59; 15,clone52; 18,clone11 and 20 in Table 3) showed no homology to any known sequences and seven other sequences (No7; 12,clone45; 13; 14,clone4; 15,clone60 and 18,clone6) displayed faint homology to several bacterial, plant, Drosophila, or mammalian genes. The very low homology does not allow a reliable identification of these candidates. One candidate (No12, clone47) has high homology with a rat EST clone, and another (No17, clone65) displayed significant homology with murine RON receptor tyrosine kinase, a member of the hepatocyte growth factor receptor family. RON receptor tyrosine kinase plays an important role in early mouse development and mediates the activation of macrophages, as well as apoptotic and growth signals (Iwama et al., 1996; Muraoka et al., 1999). The differential expression of RON receptor tyrosine kinase in three brain regions examined may reflect their differential susceptibility to apoptotic signals, leading to region-specific neurodegeneration, a well-known and largely unexplained feature of numerous brain

pathologies. This, however, deserves additional experiments.

An additional signal was expressed at higher level in the NAcc compared with two other brain regions. This pattern was not reproduced in triplicate experiments convincingly enough, and for this reason the signal is not mentioned in the table. However, it has been sequenced and strong homology was found to cysteine sulfinate decarboxylase, the key enzyme in taurine biosynthesis (Eppler et al., 1999). Taurine is a sulfur-containing amino acid that is found in abundance in mammalian tissues, including the brain. Many biological roles have been proposed for this amino acid, including in oxidative stress and cytotoxicity, and its differential expression in three brain regions could again account for differential susceptibility to neurodegenerative processes.

Table 3. Differential display 2nd set signals with only regional differences (not modified by cocaine treatment)

No	*XAp*	*Size*	*s-H*	*s-Acc*	*s-Str*	*I*	*II*	*III*	*Identification*	*Homology%*
					DLPC					
1	1	320	+	-	-	-	+	+	-	
2	1	70	-	-	+++	+	+	+	-	
3	2	130	+	++	++	+	+	+	-	
4	2	125	+	++	++	+	+	+	-	
5	10	220	++	-	-	+	?	+	-	
					DLPA					
6	2	240	+++	+	++	-	+	+	-	
7	2	400	+++	+	++	+	+	+	Mouse IgE-binding lectin gene exons 1-4 (35) Human DNA sequence from cosmid L21F12, Huntington's disease region (42)	82 % (29/35) 77 % (34/44)
8	2	320	+	-	+	?	+	+	-	
9	6	320	++	-	++	+	+	+	-	
10	6	550	++	+	+	+	+	+	-	
11	6	260	-	-	+	+	+	+	M.musculus HP1-BP74 protein	77-86 % (41/53-105/122)
12	8	320	+	-	+	+	+	+	None (44) E.coli xylose-proton symport gene, complete cds and maltose transport gene, 3' end (45) R.norvegicus cDNAclone UI-R-AO-aj-b-07-0-UI 3' (47)	 70 % (39/55) 100 % (292/292)
13	8	160	+	-	-	+	+	+	C.elegans cosmid Y51A2D (8)	88 % (31/35)
14	10	160	-	++	++	+	+	+	D.melanogaster DNA sequence (4) None (59)	75 % (34/45)
15	12	460	+	++	++	-	+	+	None (52) A.thaliana transcribed sequence (60)	 64 % (54/84)
16	12	110	-	++	++	+	+	+	-	
					DLPG					
17	1	230	++	-	+	-	+	+	M.musculus mRNA for RON receptor tyrosine kinase (65)	95 % (182/191)
18	1	190	++	-	-	?	+	+	C.acetobutylicum sigE and sigG genes for sigma factor E and sigma factor G (6) None (11)	66 % (38/57)
19	6	120	++	+	++	+	-	+	-	
20	13	280	+	-	-	+	+	+	None (40)	

XAp is the arbitrary primer used in combination with particular DLP; s: saline, c: cocaine; H: hippocampus, Acc: nucleus accumbens, Str: lateral striatum; I, II and III correspond to triplicate DD experiments; the number in parenthesis in the column "Identification" corresponds to a particular clone or clones sequenced for each fragment (see text for details)

2.1.3. Analysis of signals modified upon cocaine treatment

We further focused on signals differentially expressed upon cocaine treatment. We found 20 candidates with expression modified upon cocaine, up-regulated in 60% of the cases in a region-specific fashion (Table 4). The majority of the signals were identified with DLPA set of oligo(dT) primers. Some representative DD gels from these candidates are presented on Figure 28 and Figure 29. In most cases the differential expression was reproduced in triplicate experiments. One example of reproduction of results is shown in Figure 28. It can be clearly seen that both the overall pattern of expression as well as the differential expression of three particular fragments (No16-18 in Table 4) remained almost identical in the two independent experiments. No1 (No16 in Table 4) is present only in the cAcc; No2 (No17 in Table 4) is up-regulated in both NAcc and LStr upon cocaine treatment; No3 (No18 in Table 4) is down-regulated in cHipp. Figure 29 shows the DD experiment made with arbitrary primers XAp1,2,4 and 15 and DLPA set. No4 and 5 (No13 and 14 in Table 4) are up-regulated in the Hipp upon cocaine treatment; No6 (No11 in Table 4) is up-regulated in both cNAcc and cLStr, No7 (No12 in Table 4) is over-expressed in all three brain regions upon cocaine treatment and No8 (No19 in Table 4) is down-regulated in the cHipp and the cNAcc.

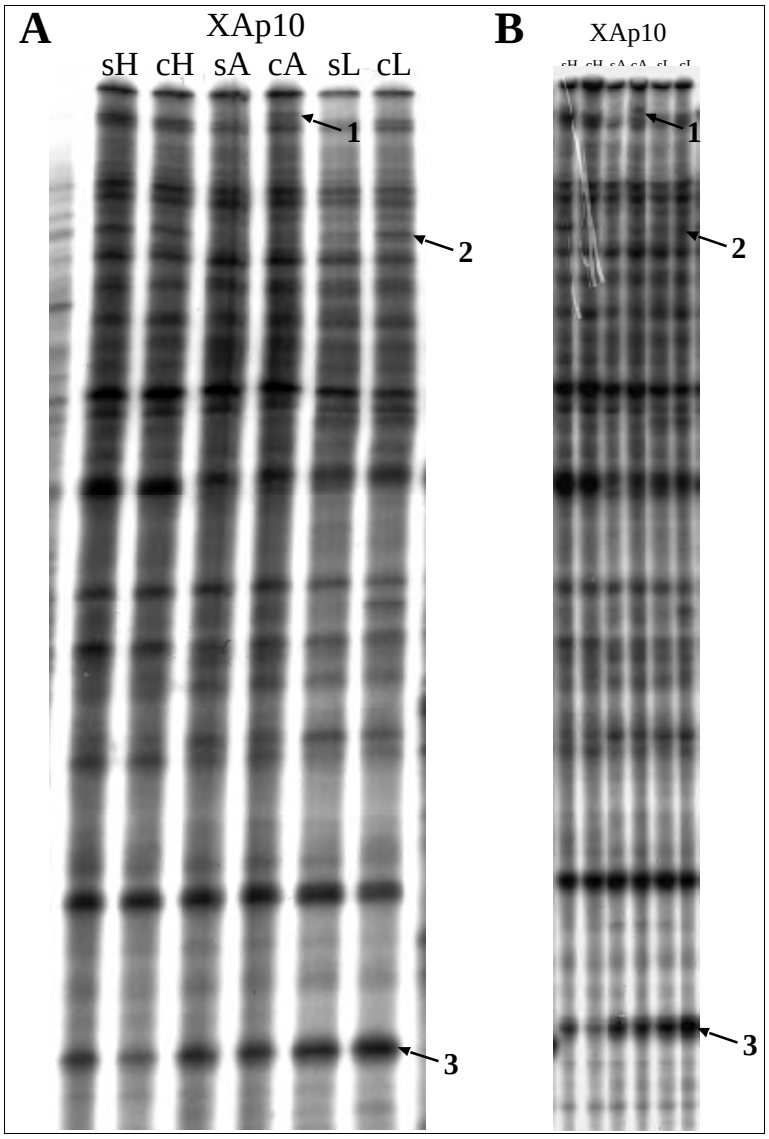


Figure 28. A and B: duplicate differential display experiments performed on cDNAs from three brain regions with DLPA oligo(dT) primer set in combination with arbitrary primer XAp10. Differentially expressed band are indicated by numbers. 1,2 and 3 correspond to N16,17 and 18 in Table 2, respectively. s: saline, c: cocaine, H: hippocampus, A: nucleus accumbens, L: lateral striatum.

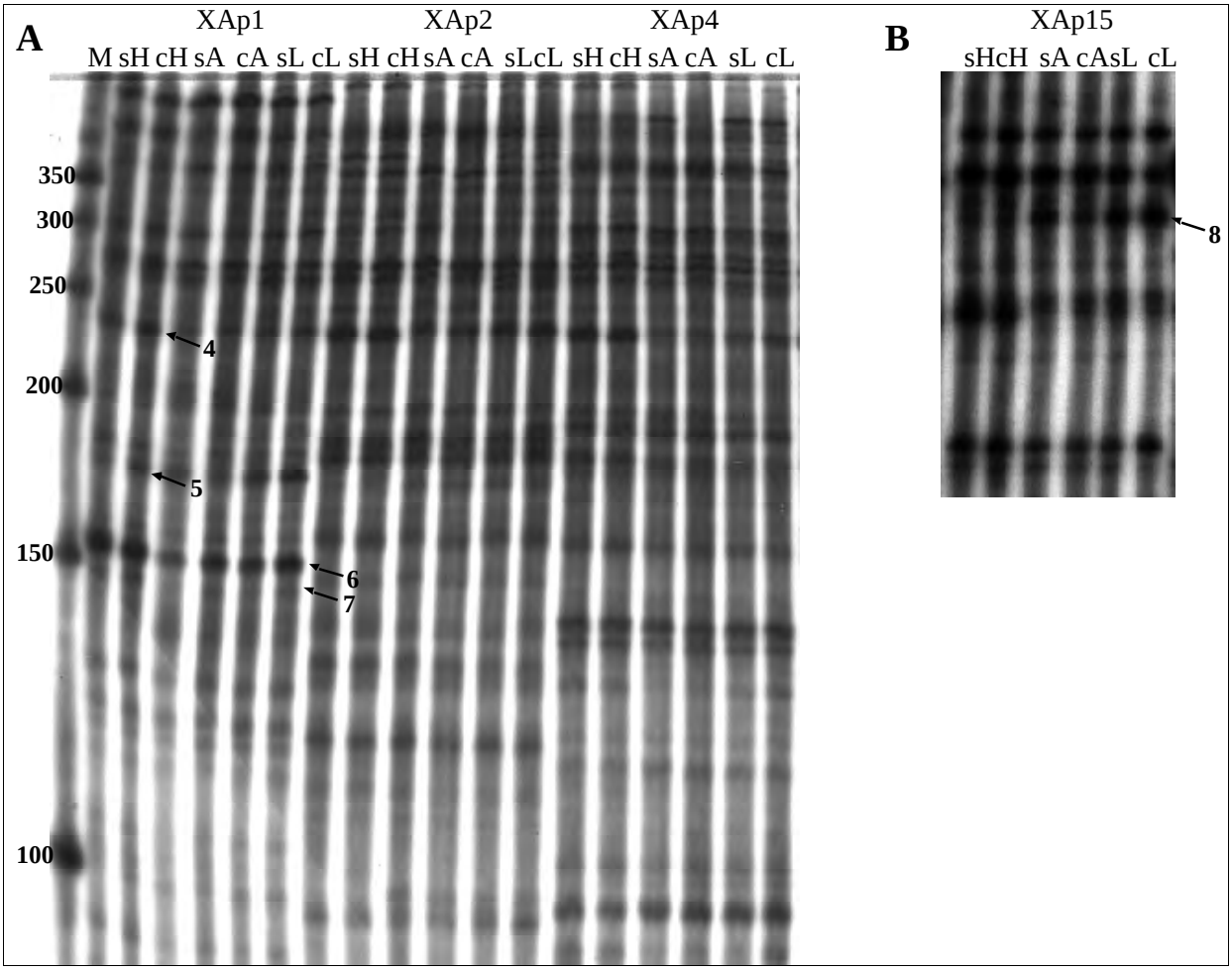


Figure 29. Differential display experiments performed on cDNAs from three brain regions with DLPA oligo(dT) primer set in combination with arbitrary primers XAp1,2,4 (A) and XAp15 (B). Differentially expressed band are indicated by numbers. 4,5,6,7 and 8 correspond to N13,14,11,12 and 19 in Table 2, respectively. s: saline, c: cocaine, H: hippocampus, A: nucleus accumbens, L: lateral striatum.

Seven fragments (No2,3,9,13,14,15 and 18 in Table 4) showed changes in expression only in one region, five of them (No2,3,13,14 and 18) in the Hipp. The expression of thirteen other fragments was modified upon cocaine treatment in several regions simultaneously. Nine out of these 13 fragments displayed a unidirectional (either up- or down-regulation) change in all regions, for example No1 was up-regulated in both NAcc and LStr. Four other fragments (No5,7,10 and 16 in Table 4) display changes in the Hipp which were opposite to the changes found in one or both striatal subregions. For 7 fragments (No1,4,6,10,11,12 and 17 in Table 4), i.e. one third of the signals, the changes were simultaneous and unidirectional in the entire striatum (NAcc and LStr).

We found 14 signals with a level of expression modulated in the Hipp, even more than for the LStr or the NAcc (No11 and 12 respectively).

From the analysis of gene expression patterns, as described prior to sequencing in the Hipp and the striatum, we could conclude that both the basal level of gene expression and the type of changes in response to cocaine treatment are similar in the striatum subregions and distinct in the Hipp, suggesting that the gene expression pattern is a valuable marker of a particular brain nucleus.

One half of the band excised from DD gel could be efficiently reamplified and further processed for direct sequencing or cloning. Only 3 fragments excised from the gel (No11,14 and 16 in Table 4) were homogeneous enough and could be sequenced directly after reamplification without cloning. The other fragments were cloned and the clones were further classified according to restriction analysis as described before for region-specific fragments. Sequences were submitted to Genbank for homology search and these results are summarized in Table 4. The majority of differentially expressed signals were identified with DLPC and DLPA sets of oligo(dT) primers. Five fragments (No10;11;13,clone30; 14;16 in Table 4) have strong homology to known mRNA sequences, whereas five clones (No12; 13,clone27; 15; 18 and 20) are homologous to mammalian ESTs. One of the known proteins, CD81, will be discussed in more detail later in the RESULTS, whereas the other known candidates and their possible relevance in terms of drug action, will be treated in the DISCUSSION section.

Table 4. Signals induced by cocaine treatment

N°	*XAp*	*Size*	*s-H*	*c-H*	*s-Acc*	*c-Acc*	*s-Str*	*c-Str*	*I*	*II*	*III*	*Identification*	*Homology, %*
DLPC													
1	1	160	++	++	-	+	-	+	+	+	+	-	
2	1	190	++	+++	+	+	+	+	+	+	+	-	
3	1	120	++	-	++	++	-	-	-	+	+	-	
4	1	360	+++	+++	+	++	+	++	+	+	+	-	
5	2	210	++	-	-	+	++	+	-	+	+	-	
6	4	350	++	+	++	+	++	+	+	+	+	-	
7	6	200	-	+++	-	-	+	++	+	+	+-	-	
8	12	180	+	-	++	+	++	++	+	?	+	-	
9	12	100	+++	+++	+	++	+	+	-	+	+	-	
10	14	245	+	++	+-	-	++	+	+	+-	+	R. norvegicus sodium bicarbonate cotransporter mRNA (18)	99 % (160/161), score 311
DLPA													
11	1	160	+++	+++	+	++	+	++	+	+	+	Rat neuronal olfactomedin-related protein or pancortin	100 % (107/107)
12	1	150	+	++	-	+	-	+	?	+	+	Human cDNA clone IMAGE:2755781 3' (9')	93 % (27/29), score 21
13	1	240	++	+++	+	+	+	+	?	+	+	M. musculus cytochrome c oxidase subunit VIIc (Cox7c) mRNA (30, prominent sequence) Sugano mouse kidney mkia M. musculus cDNA clone IMAGE:2520223 5' (27)	90 % (171/190), score 114 89 % (59/66), score 38
14	1	180	+	++	+	+	+++	+++	+?	+?	+	R. rattus guanine nucleotide-releasing protein or mss4 mRNA	100 % (134/134), score 266
15	8	190	-	-	-	-	-	+	-+	+	+	Chromosomic repeat region / rpt family "B2" or Normalized rat embryo, cDNA clone REMDY11 3' end (13, prominent sequence) R. norvegicus cDNA clone RGIDY49 5' end (11)	Score 194 93 % (134/143), score 200 87 % (35/40), score 40.1
16	10	600	+	-	-	++	-	-	+	+	+	R.norvegicus target of the antiproliferative antibody or CD81	98 % (341/346)
17	10	390	++	++	-	+	+	++	+	+	+	-	
18	10	120	++	+	+++	+++	+++	+++	+	+	+	M. musculus cDNA clone DEBT-24 (22)	100 % (24/24), score 48.1
19	15	260	++	+	++	+	+++	+++	-	+	+	-	
DLPG													
20	9	160	+	++	-	-	+	++	-	+	+	Adult female mammary gland M. musculus cDNA clone D730035C01 (72)	100 % (19/19), score 38.2

20 signals differentially expressed in the Hipp, the NAcc and the LStr upon cocaine treatment as found by differential display on mRNA template by combinations of 3 sets of oligo(dT) primers and 20 arbitrary primers in three independent experiments are presented. s: saline, c: cocaine. For other abbreviations see Table 3. The prominent sequence stands for the clone whose sequence was shown by restriction analysis to be predominant for a particular fragment. Fragments without sequencing results are candidates that could not be efficiently reamplified and proceeded for the subsequent analysis.

2.2. Changes in gene expression upon cocaine treatment in the tegmentum

A DD was performed with untreated and cocaine-treated tegmentum according to our protocol with some minor modifications. Poly(A) RNA was used instead of total RNA for the reverse transcription reaction. DD was performed in duplicate starting from two independent reverse transcription reactions. Again some arbitrary primers did not detect expression differences. In both DD experiments no differential expression was found with the arbitrary primers XAp 3,11,13,16 and 18.

We found 23 candidates whose expression was modified upon cocaine, up-regulated in 7 cases (Table 5). The fragment size was in the range of 105-460bp. All differences have been confirmed in duplicate experiments except for No20. High heterogeneity was found in each band isolated from the DD gel, however, but one sequence was predominant for each signal (for example, type I for fragment No1 or type II for fragment No5). 15 fragments were sequenced, and homology search was performed in Genbank. Results are summarized in Table 5. Fragments No4/II,10,12,13 and 21 have strong homology to known sequences, and will be discussed in more detail in the DISCUSSION section. Fragments No2,4/I,8,9,14,16,17 and 19 are homologous to ESTs or genomic sequences. In this display we found a number of mitochondrial sequences (No9,12,21 in Table 5). One of them (No12), corresponding to NADH dehydrogenase subunit 6, has already been described previously to be up-regulated by cocaine self-administration in the same brain region (Couceyro et al., 1997).

In some cases not only the up-regulated band but also the corresponding piece of gel from the lane of comparison were excised from the DD gel for the reamplification. Both were subsequently cloned and analyzed. This type of analysis however did not help, as we expected, to distinguish between truly differential signal and contaminating sequences present at equal amounts in both conditions. In four cases chosen for such analysis (pairs N1/2,4/5,7/8 and 21/22 in Table 5) we could not detect any clone absent in the control lane which would be then considered for sequencing. This is probably due to the reamplification PCR reaction, which is made under conditions permissive to achieve the saturation, so that even if the differences were initially present in two bands, they are not detectable anymore after this PCR.

Table 5. Changes in gene expression upon cocaine treatment in the tegmentum

N°	*DLP*	*XAp*	*Size*	*Ts*	*Tc*	*N° of clones*	*RsaI restriction types*	*Identification, Genbank accession number and homology score*
1	A	2	195	+++	+[1]	15	I (10), II (1), III (2), IV (1), V (1)	-
2	A	2	195	+++[1]	+	15	I (14), II (1)	I: m.musculus mammary gland cDNA clone 3' end, AW990522, 54
3	A	2	155	+	-	-	-	-
4	A	4	235	++	+[2]	17	I (3), II (10), III (1), IV (1), V(2)	II: r.rattus pyruvate dehydrogenase E1 alpha form 1 subunit, Z12158, 361; I: rat PC-12 cells cDNA clone 5' end, AA685854, 307
5	A	4	235	++[2]	+	15	II (9), III (1), V (2), VI (3)	II: the same sequence as 4/II
6	A	4	165	+	-	15	I (9), II (4), III (2)	-
7	A	4	120	++	+[3]	11	I (5), II (2), III (1), IV (1), V (1), VI (1)	I: the same sequence as 8/I
8	A	4	120	++[3]	+	10	I (6), II (4)	I: rat PC-12 cells cDNA clone 5'end, H32747, 109; II: h.sapiens cDNA, BE094563, 38.2
9	A	5	175	-	++	11	I (7), II (1), III (1), IV (1), V (1)	I,IV,V: Sprague-Dawley rat female mitochondrial genome unknown protein, J01435, 236
10	A	5	170	-	+++	8	I (8)	I: r.norvegicus mRNA for ecto-apyrase, Y15685, 232
11	A	5	145	-	++	12	I (1), II (11)	II: the same sequence as 12/I
12	A	5	140	-	+++	13	I (13)	I: r.norvegicus NADH dehydrogenase subunit 6, X14848, 172
13	A	5	110	-	++	11	I (11)	I: r. norvegicus cDNA clone 3' similar to sensory neuron synuclein, AI070517, 310
14	A	5	105	-	++	16	I (15), II (1)	I: rat/SV40 recombination junction clone, K02744, 42.1
15	A	5	178	+	-	4	I	-
16	A	5	148	++	-	15	I (13), II (2)	I: r.norvegicus cDNA clone 3', AW522220, 184
17	A	5	120	++	+	8	I (7), II (1)	I: h.sapiens cDNA clone 3', H22951, 131
18	A	12	460	+	-	1	I (1)	-
19	A	7	160	++	-	15	I (9), II (3), III (2), IV (1)	I: m.musculus chromosome 7 clone, AP001295, 172
20	A	8	320	+	-	2-3	not-done	-
21	A	9	240	++[4]	+	8	I (8)	I: r.norvegicus cytochrome c oxidase subunit III, NC_001665, 381
22	A	9	240	++	+[4]	11	I (6), II (3), III (1), IV (1)	-
23	G	17	175	+	-	-	-	-
24	G	19	155	-	+++	-	-	-
25	G	19	145	++	+	-	-	-
26	G	20	230	+	-	-	-	-
27	G	20	115	+	-	-	-	-

Tag N° corresponds to the number of signal on gel, DLP and XAP are the oligo(dT) and arbitrary primers used respectively, T: tegmentum, s: saline, c: cocaine; types are based on restriction analysis with RsaI, types numbers are labeled by roman numbers, the number of clones in each type is indicated by Arabic numbers in the parenthesis. Subscript numbers indicate fragments for whose the bands from both the control and the treated lanes were isolated from DD gel.

2.3. Identified genes regulated by cocaine treatment

Identified candidates, whose expression was modified by cocaine treatment are summarized in Table 6. Five fragments (No2,3,4,7 and 8) were up-regulated, two fragments (No6 and 9) were down-regulated and two fragments (No1 and 5) were either up- or down-regulated depending upon particular brain region, by cocaine treatment. Three candidates are transmembrane proteins (No1,5 and 7), three others are mitochondrial (No3,8 and 9), two candidates are intracellular molecules (No4 and 6) and for one protein (No2) the localization has not been established so far.

Table 6. Identified genes regulated by cocaine treatment

N°	Protein	Changes in mRNA expression upon cocaine treatment
1	Sodium bicarbonate cotransporter (NBC)	Up-regulation in the Hipp, down-regulation in the striatum
2	Neuronal olfactomedin-related protein or pancortin	Up-regulation in the striatum
3	Cytochrome c oxidase subunit VIIc (Cox7c)	Up-regulation in the Hipp
4	Guanine nucleotide-releasing protein or mss4	Up-regulation in the Hipp
5	Target of the antiproliferative antibody (TAPA-1) or CD81	Down-regulation in the Hipp, up-regulation in the NAcc
6	Pyruvate dehydrogenase E1 alpha form 1 subunit	Down-regulation in the tegmentum
7	Ecto-apyrase	Up-regulation in the tegmentum
8	NADH dehydrogenase subunit 6	Up-regulation in the tegmentum
9	Sensory neuron or γ-synuclein	Up-regulation in the tegmentum
10	Cytochrome c oxidase subunit III (Cox3)	Down-regulation in the tegmentum

Some of the sequences were proceeded to further analysis, e.g. quantitative RT-PCR and *in situ* hybridization. Experiments with CD81 and γ-synuclein are described in the next chapters. In addition, behavioral and neurochemical experiments with CD81-KO mice have been performed. The possible relation of other candidates to cocaine function are discussed in the DISCUSSION section.

3. *Confirmation of differential display findings*

The steps between initial DD screening and subsequent functional analysis of interesting candidates include the necessary confirmation of results. The first step in any case is the band reamplification. This can be followed by cloning, restriction analysis and sequencing of the fragments. It is not strictly indispensable at this point, but facilitates substantially the subsequent analysis by eliminating the irrelevant candidates. There are a number of methods of confirmation of DD, which corresponds to the general methods of RNA quantification. Probably, the best method in terms of sensitivity and reliability is Northern blot, however it requires large amounts of RNA, an important limitation for investigating gene expression in the brain. Other methods include reverse Northern blot (dot blot, microarray or plaque assay), RNase protection assay, *in situ* hybridization and quantitative RT-PCR. All these methods were tested in the present study with variable success and will be discussed now.

3.1. Reverse Northern blot

Reverse Northern consists in applying the cDNA fragments of interest (reamplification products or cloned fragments in this case) on a solid support, in most cases nylon membrane or glass, followed by hybridization with labeled total cDNAs pool of the examined tissue. Clones may be applied on the solid support by manual pipetting on the nylon membrane (dot blot), transfer of growing colonies from transformed clones onto the nylon or nitrocellulose membrane (plaque assay) or machine application of a tiny amount of cDNA on, for example, glass membrane (microarray). In our work we used both dot blot and plaque assay, with probes labeled by incorporation of either radioactively or DIG-labeled nucleotide in the reverse transcription reaction. In the latter case chemiluminescent detection was performed upon hybridization.

Several problems were noted with this method, making the interpretation of the results unreliable. First, and major problem, a persistent and strong signal appeared with the negative control (empty vector) in hybridization. Very often the signal was virtually indistinguishable from positive controls used for normalization (beta-actin, GAPDH etc.). Clearly the presence of such a signal casts doubt on any other data from the same experiment. The second problem concerned the high variability of signal intensities, with no correlation to the known level of expression of a

particular gene in the cell. This is an inherent problem of the method, due to the fact that all experiments are performed under a standard hybridization protocol, independently of particular sequence requirement. Finally, the method is not very sensitive. Therefore, the method of choice in our study could be glass microarrays, containing genes of interest, including all signals found by DD as well as other candidates potentially interesting in respect to drug addiction. Membranes have been prepared in this way and will be soon subjected to hybridization with fluorescent probes.

3.2. Ribonuclease protection assay

Ribonuclease protection assay (RPA) consists in the hybridization of RNAs with a radioactively labeled riboprobe prepared from the clone of interest. The riboprobe contains an additional fragment, not related to the sequence of interest (a fragment of the cloning vector, for example). The hybridization is followed by treatment with single-strand specific RNase which will leave the hybrid undigested, resulting in the formation of a protected fragment. The mixture is then separated on polyacrylamide gel. The intensity of the protected fragment band is analyzed by autoradiography and corresponds to the relative concentration of the fragment of interest in a particular tissue sample. The method is highly specific, but very tedious, requires high amounts of radioactive material, and a limited number of samples may be processed simultaneously. Probably the major problem that we faced with this method, is its extreme sensitivity towards the quality of the RNA used. The isolation of RNA from brain or other tissue is more tricky compared to cultured cells, because the RNA is instantaneously exposed to tissue RNases and some degradation always occurs. So far, we were not able to adapt RPA for confirmation of DD results in brain samples.

3.3. In situ hybridization

In situ hybridization consists in hybridization of tissue sections with radioactively or non-radioactively labeled oligo- or riboprobes, followed by evaluation by light microscopy. In our study we used DIG-labeled riboprobe. This method has been particularly useful for the description of the distribution of a particular signal, but it is not suitable for quantification of the level of expression. An additional step (compared to radioactivity) includes incubation with antibody, which must also be normalized. Radioactive *in situ* hybridization is a suitable method among other methods for RNA quantification, allowing for exact spatial resolution of the signal, which is not accessible by other methods. However, it is certainly not a method of choice when screening of multiple candidates from a DD, because it is laborious, requests a large amount of animals for reliable statistical analysis of the results, and requires careful interpretation of the data, including the precise neuroanatomical identification of the brain regions examined.

3.4. Quantitative RT-PCR

The quantitative RT-PCR is a method allowing for very sensitive and reproducible quantification of a particular probe. It consists in amplifying of fragment of interest with sequence-specific primers in parallel with amplification of a house-keeping gene as positive control for the normalization of the results. Quantification is performed either on the final amount of the amplified product, or on the time-point when the amplification passes a certain threshold (real-time PCR). The first method is not very reliable for quantification, whereas the second procedure was virtually inaccessible for a long time for many laboratories, given the high cost of fluorescent probes used in the reaction. An adaptation of the method, using the SYBR Green dye which specifically incorporates into the double-stranded DNA sequences has been used in our study to confirm the CD81 up-regulation in the NAcc of cocaine-treated brain (see Section III.4).

4. *Cocaine-induced expression of the CD81 in the NAcc (Annexes II & III)*

As mentioned before, CD81 was found by DD to be up-regulated in the NAcc of rats following acute treatment with cocaine and drug withdrawal. The message was present neither in the NAcc of saline-treated animals nor in lateral striatum at any condition. CD81 is a tetraspanin transmembrane protein, known to be expressed in astrocytes and involved in cell adhesion. In the following paper we confirm our data by quantitative real-time RT-PCR and describe the expression of CD81 in the adult rat brain (Annex II). Quantitative PCR was performed with 28S rRNA as a positive control for normalization. CD81 was up-regulated after cocaine treatment with a 4.6 fold raise in the expression in the NAcc.

By means of *in situ* hybridization, we showed CD81 expression in many glial structures (choroid plexus, ependyma, glia limitans) in accordance to previously published data (Sullivan et al., 1998). However we could not confirm the exclusivity of such glial-specific patterns. Many groups of CD81-positive cells (paraventricular hypothalamic nucleus (PVH), supraoptic nucleus (SO) or diagonal band of Broca) showed a pattern of staining that cannot be attributed to glial cells.

We also show the expression of CD81 in brain regions functionally related to the regulation of cardiovascular function and fluid homeostasis. The brain systems regulating these functions include a sensory part composed of the choroid plexus, the subfornical organ, the vascular organ of lamina terminalis as well as the median preoptic nucleus and a motor or effector part, represented by the magnocellular, vasopressin- and oxytocin-secreting, cells of PVH and SO in the hypothalamus. All these regions, as well as other regions projecting to PVH and SO, including the bed nucleus of the stria terminalis (BST), DB and medial preoptic nucleus, were also positive for CD81.

In addition, CD81 showed similar brain distribution to galanin, and, to a lesser extent, to vasopressin. The co-distribution was especially striking in the magnocellular

cells of the PVH and in the anterodorsal thalamic nucleus. Interaction of CD81 with the Gal pathway could alter dopaminergic responses and influence the reward pathway. These findings, added to previous data, suggest a connection between the brain reward pathways and centers regulating endocrine and autonomic functions, and may show a relation of the neurochemical, behavioral and somatic consequences of drug abuse.

The CD81 is known to form complexes with integrins, which mediate probably all CD81 cellular effects. By comparing our data with published results on brain integrin subunits, we could conclude that CD81 could interact with both α3β1 and α6β1 heterodimers, at least in magnocellular neurons of the hypothalamus and in the SO.

The second paper presents behavioral and neurochemical data on CD81-knockout mice (Annex III). The behavioral paradigms employed for the first objective were locomotor activity (used to assess the stimulant properties of the drug) and place preference conditioning (used to assess the reinforcing efficacy of the drug). The C57BL6 mice were used as controls in all experiments.

In place preference experiments the animals received the saline in the lined and the cocaine in the bare chamber, respectively. Both before and after conditioning their preference for the particular chamber type was tested by measuring the relative time spent in each chamber. Both male and female control mice spent more time in the bare, cocaine-paired chamber, after conditioning. This was not the case for CD81-deficient males. However this difference was at least partially smoothed by the fact that male mutant mice showed the higher preference for the lined chamber before conditioning as compared to wild-type littermates. Female CD81-deficient mice did not display the preference for the bare, cocaine-paired chamber, after the conditioning, except for the first day of trials.

Male C57BL6 mice were more active than male CD81-deficient mice following low doses of cocaine. When the dose was increased, this difference in the locomotor activity was no more evident. The results with female mice are more difficult to interpret. Whereas the activity curve of wild-type animals is very similar with the one for male controls, CD81-deficient mice show a decreased activity at 0.1 and 3 mg/kg of cocaine, highly increased response upon the injection of 1mg/kg cocaine and no changes upon the highest dose administrated, 10 mg/kg.

Finally, neurochemical changes in CD81-deficient mice brain were observed. In the NAcc of male knockouts, a significant increase in the concentration of dopamine, as well as dopamine metabolite DOPAC, was detected. These changes were region-specific (no differences in the LStr), neurotransmitter-specific (no changes in the level of serotonin and its metabolites), and sex-specific (no changes detected in female CD81-deficient mice for any of parameters measured in the study).

These data demonstrate the importance of the CD81 for cocaine action, and more precisely, its sex-specific function in drug addiction. These observations are consistent with previously discussed involvement of CD81 in hypothalamic function, a brain structure mostly responsible for the hormonal, sex-specific differences in neuroendocrine regulation.

In addition, very recently a series of experiments also demonstrated the altered response of CD81-deficient mice to amphetamine, both in regard to dopamine and serotonin metabolism. These effects were again sex-specific.

5. *Cocaine-modulated expression of γ-synuclein in the tegmentum (Annex IV)*

Another candidate, rat sensory neuron synuclein, persyn or γ-synuclein, was also found by DD to be up-regulated in the tegmentum of rats following acute treatment with cocaine and drug withdrawal. This candidate displayed a pattern of "all-or-none" regulation in the tegmental area. The homology was found in the 3' region of the mRNA sequence, which is not included in the high homology region defining the synuclein family, thus the un-equivocal identification of the signal was possible.

Synucleins are small (120-140 aa) proteins sharing structural resemblance with apolipoproteins (Clayton and George, 1999). They belong to a family of four members, α-, β-, γ-synucleins and synoretin, from which the γ-synuclein is the most distinct member. It lacks a conserved C terminal domain of α- and β-synucleins and displays different spatial and temporal expression, being distributed diffusely throughout the cell body and axons, at least in peripheral neurons, and not in the presynaptic terminal. γ-synuclein stimulates breast cancer invasion and metastasis (Jia et al., 1999) and accumulates in axons of patients with Parkinson's disease or Lewy body dementia (Galvin et al., 1999). This last feature makes γ-synuclein a close relative to α-synuclein, a protein involved in the pathogenesis of several neurodegenerative diseases. For this reason we investigated in more details the distribution of α-and γ-synucleins in the adult rat brain, with a view of better understanding their respective functions.

In general α-synuclein displayed a very characteristic pattern of expression, confined to specific nuclei, whereas γ-synuclein was more widely expressed throughout the brain. The two proteins displayed a complementary pattern of expression all over the cortex. Both ependymal ventricular lining cells and the choroid plexus were positive for γ-, but not for α-synuclein. α-synuclein was expressed mostly in olfactory areas, whereas γ-synuclein was more present in the basal ganglia. Both mRNAs were present in the hippocampus at a relatively high level. In both thalamus and hypothalamus, very specific nuclei were positive for α-synuclein, whereas γ-synuclein was largely presented in both regions. The signal in the paraventricular nucleus was mostly confined to subsets of neurons involved in the control of the autonomic nervous system. α-synuclein expression is highly correlated with TH expression, whereas the correlation was poor for γ-synuclein.

In conclusion, the two members of the synuclein family showed a distinct distribution pattern, suggesting distinct functions for these highly related proteins. In

addition, γ-synuclein modulation by cocaine opens new insights on the correlation between the neurodegenerative disorders such as Parkinson's and drug addiction, both related to the dopaminergic system. However, very recent experiments with quantitative RT-PCR did not confirm the original DD finding. Therefore, the precise function of synucleins in cocaine action needs to be further determined.

6. *Changes in CART expression upon cocaine treatment*

We were interested to check whether other molecules, known to be induced by acute cocaine, also display an altered expression in our conditions. In our protocol gene expression is measured 24 hrs after the last injection, therefore we do not expect to find changes in the expression of immediate early genes or related molecules, which display a high, but transient, burst of induction. On the other hand CART, a molecule related to neuroplasticity events in the DAergic system might be regulated under our conditions. As a matter of fact *in situ* hybridization revealed CART up-regulation in the NAcc upon cocaine treatment (Figure 30). The signal is particularly strong in the shell of NAcc.

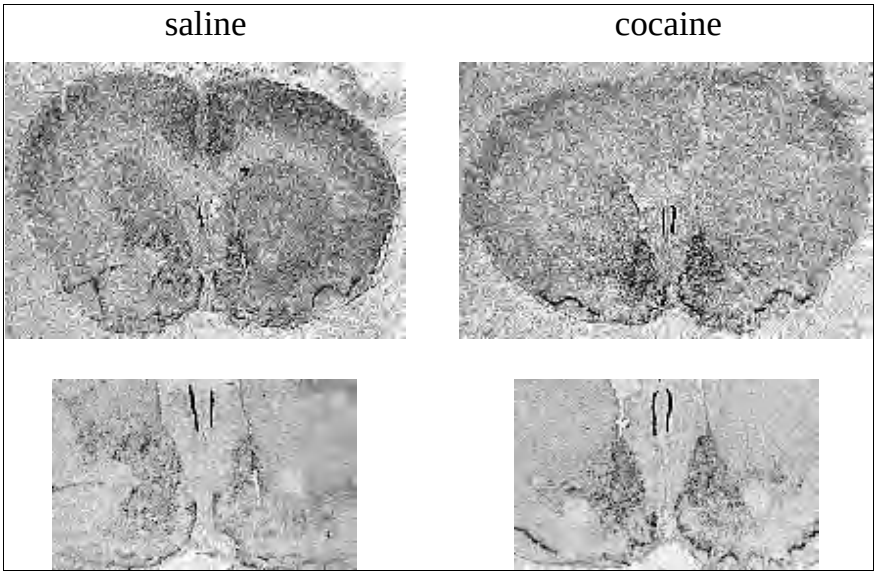


Figure 30. Up-regulation of CART in the NAcc of cocaine-treated rats

7. *Semaphorin cloning and targeted display*

7.1. The identification of new members of rat semaphorin family.

Nine identified candidates display changes in expression after cocaine treatment (Table 6). In addition, two axon guidance molecules, ephrin-B2 and Eph-A5, have been described to be up-regulated by cocaine using the same protocol of animal treatment as used in this study. Therefore we became interested at investigating the expression of other families of axon guidance molecules in cocaine-treated brains, e.g. the semaphorins. Semaphorins are a group of axon guidance molecules (for reviews see: (Artigiani et al., 1999; Raper, 2000; Yu and Kolodkin, 1999)) found in different organisms, including drosophila, grasshopper, chicken and mice. About 20 members of the family have been identified. Despite the intensive efforts in cloning new members of the semaphorin gene family, very little orthologues are known for the rat. From 19 potential semaphorins (one for each subclass) only four were identified in the rat at the time of development of a semaphorin nomenclature (1999). Therefore, we decided to clone new rat semaphorins, using the method of targeted RT-PCR. This method takes advantage of the presence of a highly conserved Sema domain of 500 amino acids in each family member (Figure 31).

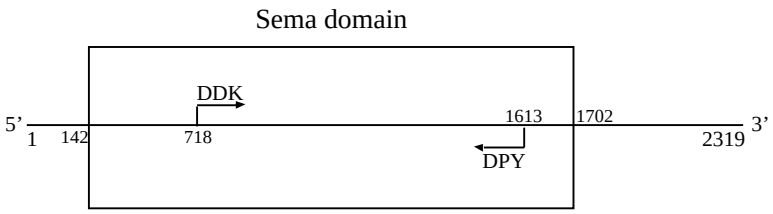


Figure 31. The relative position of Sema domain and semaphorin-specific degenerate primers DDK and DPY within the semaphorin sequence (this example is for rat Sema3A (R-SemaIII))

Briefly, two degenerate primers are designed within the most conserved part of the Sema domain, based on amino acid sequence. The degeneracy of a given primer is determined by multiplying the number of possible nucleotides at each position. Degeneracy at the 3' terminus are avoided, because single-base

mismatches may obviate extension, and the last base of the terminal codon is omitted. We have chosen the pair of degenerate primers DDK and DPY previously used for the cloning of avian semaphorins (Luo et al., 1995) (Figure 31 and Figure 32). DDK and DPY primers code for 8 and 7 amino acids respectively, and their design matches all requested criteria. They were initially designed based on conserved protein sequences from two avian semaphorins. We performed the amino acid alignment of the Sema domain of different rodent semaphorins and we could establish that DDK and DPY primers should be specific enough for cloning of new rat semaphorins. The alignment as well as primers nucleotide sequence are shown on Figure 32.

```
mSema-A             -QNPSLRTEPHDSRWLN-EPKFVKVFWIPESENP---DDDKIYFFFRESAVEAAP 254
mSema-B             -SHPVLKTDIFLR-WLHADASFVAAIPS----------TQVVYFFFEETASEFD- 255
mSema-C             -SSRPTKTESSLN-WLQ-DPAFVASATSPESLGSPIGDDDKIYFFFSETGQEFE- 218
mSema-D             -DHHPIRTEQHDSRWLN-DPRFISAHLIPESDNP---EDDKVYFFFRENAIGGE- 255
mSema-E             -KRMQLRTDQHNSKWLS-EPMFVDAHVIPDGTDP---NDAKVYFFFKERLTDNN- 252
mSema-F             -TLPPLRTAQYNSKWLN-EPNFVSSYDIG----------NFTYFFFRENAVEHD- 241
mSema-F-like        -THHSIKTEYLAF-WLN-EPHFVGSAFVPESVGSFTGDDDKIYFFFSERAVEYD- 247
mSema-G             -SHSPLRTEYAIP-WLN-EPSFVFADVIQKSPDGPEGEDDKVYFFFTEVSVEYE- 243
mSema-H             -KLGHIRTEHDDERLLK-EPKFVGSYMIPDNEDR---DDNKMYFFFTEKALEAE- 256
mSema-VIa           -DSPTLRTVKHDSKWLK-EPYFVQAVDY--G--------DYIYFFFREIAVEYN- 244
mSema-VIb           -DRPTLRTVKHDSKWFK-EPYFVHAVEW--G--------SHVYFFFREIAMEFN- 254
mCollapsin-1        -HHHPIRTEQHDSRWLN-DPRFISAHLIPESDNP---EDDKVYFFFRENAIDGE- 255
rCollapsin-III/1    -HHHPIRTEQHDSRWLN-DPRFISAHLIPESDNP---EDDKVYFFFRENAIDGE- 255
hSema-I             -HHHPIRTEQHDSRWLN-DPKFISAHLISESDNP---EDDKVYFFFRENAIDGE- 255
hSemaphorin-E       -KRNAVRTDQHNSKWLS-EPMFVDAHVIPDGTDP---NDAKVYFFFKEKLTDNN- 252
hSemaphorin-III     -KQTAMRTDQYNSRWLN-DPSFIHAELIPDSAE----NDDKLYFFFRERSAEAP- 255
hSemaphorin-CD100   -SHSPLRTEYAIP-WLN-EPSFVFADVIRKSPDSPDGEDDRVYFFFTEVSVEYE- 243
hSemaphorin-V       -QRPSLRTEPHDSRWLN-EPKFVKVFWIPESENP---DDDKIYFFFRETAVEAAP 255
gCollapsin-1        -HHHPIRTEQHDSRWLN-DPRFISAHLIPESDNP---EDDKIYFFFRENAIDGE- 255
gCollapsin-2        -DHHYIRTDISEHYWLT-GAKFIATFPIPDTYNP---DDDKIYFFFREISQDSS- 260
gCollapsin-3        -KRNAVRTDQHNSKWLS-EPIFVDAHVIPDGTDP---NDAKIYFFFKERLTDNS- 252
gCollapsin-5        -RMAHLRTEPDSEHLLK-EPKFVGSYMIPDNEDH---DDNKVYLFFTEKALEAE- 260
gCollapsin-3part    ------------------------------------DDKIYFFFKERLTDNS- 16
gCollapsin-4part    ------------------------------------DDKIYFFFTEVSVEYE- 16
dSema-I             ---EPLQTEQYDSLSLN-APNFVSSFTQG----------DFVYFFFRETAVEFI- 225
dSema-II            LEYKFKRTLKYDSKWLD-KPNFVGSFDIG----------EYVYFFFRETAVEYI- 259
tSema-I             ---EPQRTELSDLKQLN-APNFVNSVAYG----------DYIFFFYRETAVEYM- 228
CeSema              KSAANIRTQSYDARVLN-APNFVATFAY-----K-----EHVYFWFREIASEAID 248

mSema-A             ALGR-ACAECCLA-RDPYCAWDGS------ACTRFQPT---AK---------RRF 551
mSema-B             VYE--SCVDCVLA-RDPHCAWDPESR----LCSLLSGS--------------TKP 532
mSema-C             LYP--TCGDCLLA-RDPYCAWTGS------ACRLASLYQPDLA---------SRP 510
mSema-D             IYGK-ACAECCLA-RDPYCAWDGS------SCSRYFPT---AK---------RRT 553
mSema-E             IYGT-ACADCCLA-RDPYCAWDGH------SCSRFYPT---GK---------RRS 550
mSema-F             FHQ--TRSACIGA-QDPYCGWDAVMK----KCTSLEESLSMTQWDQSIPTCPTRN 536
mSema-F-like        KYR--FCVDCVLA-RDPYCAWNVN------TSRCVATTSGRSG---------SFL 538
mSema-G             KHG--SCEDCVLA-RDPYCAWSPA------IKACVTLHQEEAS---------SRG 541
mSema-H             MYGS-ACADCCLA-RDPYCAWDGI------SCSRYYPTGAHEK---------RRF 559
mSema-VIa           RHG--KCKKTCIASRDPYCGWVRESG----SCAHLSPL---S----------RLT 552
mSema-VIb           LYS--GCMKNCIGSQDPYCGWAPD-G----SCIFLRPG---T----------SAT 564
mCollapsin-1        IYGK-ACAECCLA-RDPYCAWDGS------SCSRYFPT---AK---------RRT 553
rCollapsin-III/1    IYGK-ACAECCLA-RDPYCAWDGS------SCSRYFPT---AK---------RRT 553
hSema-I             IYGK-ACAECCLA-RDPYCAWDGS------ACSRYFPT---AK---------RRT 553
hSemaphorin-E       IYGT-ACADCCLA-RDPYCAWDGH------SCSRFYPT---GK---------RRS 550
hSemaphorin-III     AYGA-ACADCCLA-RDPYCAWDGQ------ACSRYTAS---SK---------RRS 552
hSemaphorin-CD100   KHG--TCEDCVLA-RDPYCAWSPP------TATCVALHQTESP---------SRG 541
hSemaphorin-V       AHGR-VCTECCLA-RDPYCAWDGV------ACTRFQPS---AK---------RRF 552
gCollapsin-1        VYGK-ACAECCLA-RDPYCAWDGS------SCSRYFPT---AK---------RRT 553
gCollapsin-2        TYGK-ACADCCLA-RDPYCAWDGN------SCSRYAPT---SK---------RRA 558
gCollapsin-3        IYGT-ACADCCLA-RDPYCAWDGN------SCSRFYPT---GK---------RRS 550
gCollapsin-5        MYGT-ACADCCLA-RDPYCAWDGI------SCSRYYPTGMQAK---------RRF 562
gCollapsin-3part    IYGT-ACADCCLA-RDP---------------------------------------- 294
gCollapsin-4part    TYT--TCFDCVLA-RDPYCAW---------------------------------- 295
dSema-I             NDKITSCSECVAL-QDPYCAWDKIAG----KCRSHGAPR-WLE---------ENY 538
dSema-II            RRYD-NCFRCVR---DPYCGWDKEAN----TCRPYELD----------------- 538
tSema-I             SKT--RCKDCVEL-QDPHCAWDAKQN----LCVSIDTV---TS---------YRF 523
CeSema              QQT--SCSKCVQL-QDPHCDWDSSIARQVIVMSSLTPT---SL---------MWT 541
```

DDK amino acid sequence	D D K Y I F F F
nucleotide sequence	5'-GAY GAY AAR ATH TAY TTY TTY TT-3'
DPY amino acid sequence	D W A C Y P D
nucleotide sequence	5'-TCC CAN GCR CAR TAN GGR TC-3'

Figure 32. Conservation of SEMA domain sequence for degenerate primers DDK and DPY. Highly conserved amino acid sequences DDKIYFFF and DPYCAWD are shown in bold. The nucleotide sequences of the primers are given in the table below the alignment.

The cDNA was produced from total RNA extracted from rat brain, and PCR resulted in a fragment of the expected size of 900 bp. The specificity of the amplification was assessed with negative controls, where one of the primers was omitted (DDK). The fragment was purified from preparative agarose gel, treated with T4 DNA polymerase in order to remove the adenosine nucleotide added during the PCR step, phosphorylated with T4 polynucleotide kinase and cloned into the pBSKS+ vector, restricted with EcoRV, producing blunt ends. The transformation and plating resulted into 41 clones, which were proceeded for further analysis. A first analytical restriction was performed with BamHI and Eco0109I (Table 7).

Table 7. Restriction analysis of semaphorin clones 1-43

Clone number	BamHI restriction, bp	Eco0109I restriction, bp
1,8,12,14,30,32,33	600 and 100	300 and 150
2,4	250 and 150	400
3,38,40,43	-	400
5,13,15,17,19,25,26,31,34,41	-	-
6	-	about 10 fragments
7	-	1150, 700, 400 and 270
9,16,22,28	-	2100
18,23	500 and 300	550
20	-	3100,2000,1200 and 600
21	-	3000 and 700
24	1000	380, 250 and 100
27	260	550
29	900	380
35	-	400 and 250
36	600 and 100	330
37	-	650, 400 and 260
39	-	350
42	140	300

This primary analysis resulted in several patterns of restriction. On this basis, a first potential group of fragments, homologous in nucleotide composition, was obtained (for example, No1,8,12,14,30,32 and 33 in Table 7). A subsequent analysis with different restriction enzymes gradually narrowed the number of candidates to analyze and of resulting groups. Finally 14 different groups were identified. One or two clones from each group were sequenced, and seven clones had a significant homology with members of the semaphorin family (groups I-VII in Table 8). Six candidates were found with homologies to murine or human members of the semaphorin family, whereas one clone (group VII) had closest homology with the avian semaphorin, G.gallus collapsin-2. No mammalian orthologue of this semaphorin has been identified so far. Seven other clones (groups II-XIV in Table 8) did not carry any homology to semaphorins, but in these cases only one primer (reverse semaphorin-specific primer) participated in the PCR reaction from both sides. Finally, a group-specific restriction analysis was performed with each clone in order to confirm the homogeneity of the putative members of the same group.

Table 8. Different sequences found by semaphorin-specific RT-PCR on rat brain tissue

Groups number	*Number of clones in one group (based on restriction analysis)*	*Identification (new name in parenthesis), Genbank Accession number*	*Score (bits) and homology*
I	**8** (N° 1,8,12,14,30,32,33,36)	Mouse mRNA for M-sema F (Sema4C), S79463	1170: 790/857 (92%)
II	**8** (N° 5,9,13,16,17,22,31,34)	M. musculus mRNA for semaphorin C (Sema4B), X85992	1306: 802/847 (94%)
III	**4** (N° 3,7,38,40)	M. musculus mRNA for semaphorin IV, isoform a (Sema3F), AF080091	1328: 816/864 (94%)
IV	**1** (N°19)	M, musculus mRNA for msemK1p (Sema7A), AB017532	1279: 781/824 (94%)
V	**5** (N° 2,4,18,23,27)	M. musculus mRNA for semaphorin M-sema K (Sema3E), Z80941	829: 564/612 (92%)
VI	**3** (N° 29,35,37)	M. musculus mRNA for semaphorin M-sema G (Sema4D), U69535	1203: 783/845 (92%)
VII	**1** (N° 26)	G. gallus mRNA for collapsin-2 (Sema3D), U28240	202: 315/386 (81%)
VIII	**1** (N° 39)	R. norvegicus mRNA for cytocentrin, U82623	218: 110/110 (100%)
IX*	**1** (N° 24)	M. musculus mRNA for alpha glucosidase II alpha subunit, U92793	868: 483/498 (96%)
X*	**2** (N° 21,41)	Genomic sequence from Mouse 4, AC002108	331 (68-89%)
XI*	**1** (N° 28)	Human mRNA for adipsin/complement factor D, M84526	154: 46/65 (70%)
XII*	**2** (N° 42,43)	M. musculus gene for Klotho and secreted isoform of Klotho protein, exon 2, 3 and 4, AB010090	237: 65/87 (74%)
XIII*	**1** (N° 25)	Drosophila melanogaster cytoplasmic dynein intermediate chain gene	75 %
XIV*	**1** (N° 15)	Mouse BAC mbac20 from 14D1-D2 (T-cell Receptor Alpha Locus)	96 %

*RT-PCR fragments with reverse semaphorin-specific primer from both sides

7.1.1. Classification of the cloned semaphorins

The semaphorin family comprises 7 classes. The first two classes are found only in invertebrates, class 3 includes secreted semaphorins and classes 4-6 comprise the transmembrane family members. Class 4 contains an extracellular immunoglobulin domain and a short cytoplasmic domain whereas the characteristic feature of class 5 is the presence of seven thrombospondin repeats. Class 6 contains the longest cytoplasmic tail. Finally, class 7 also contains an immunoglobulin domain as well as a GPI anchor.

Homology search served as a criteria to assign the newly cloned semaphorins into a particular class. The very high homology of groups I-VI (at least 92% at nucleotide level) with murine semaphorins allowed for unequivocal identification of the majority of the new clones as rat orthologues of these classes. The highest homology of group VII was with avian collapsin-2 (Sema3D), but it was not very pronounced (81%) and did not allow for a more precise identification prior to isolation of complete cDNA. Human mRNA sequence for SEMA3D was not identified so far, however, the genomic sequence for Sema domain is known. On this basis, we could reconstitute the putative human SEMA3D Sema domain mRNA and amino acid sequence and align it with group VII (not shown). 94% homology at the amino acid level allowed us to unmistakably identify the group VII as a true rat Sema3D orthologue.

From this analysis we found that three novel rat orthologues of class 3 semaphorins had been cloned, one of which is actually a new vertebrate Sema3D, with a closest homology to avian SEMA3D or Collapsin-2 (group VII in Table 9). This sequence has been submitted to Genbank under Accession number AF268594. We could also clone two new and one previously identified rat orthologues of class 4 (group II, partial cDNA sequence can be found in EST sequences database) and one rat orthologue of class 7.

Table 9. Semaphorin family members

Class	Chick	Human	Mouse	Rat	Zebrafish
Sema3A / SEMA3A[1]	C-Collapsin-1 (Coll-1)	H-Sema III (H-Sema-D)	M-SemD (M-Sema III)	R-SemaIII	Sema-Z1a
Sema3B / SEMA3B		H-Sema A, H-Sema V	M-SemA		
Sema3C / SEMA3C	C-Coll-3	H-Sema E*	M-SemE	cDNA clone*	
Sema3D / SEMA3D	C-Coll-2*			**group VII***	Sema-Z2
Sema3E / SEMA3E	C-Coll-5		M-Sema H (M-Sema K*)	**group V***	
Sema3F / SEMA3F		H-Sema IV, H-SEMA3F	M-Sema IVa* and b	**group III***	
Sema4A / SEMA4A			M-SemB		
Sema4B / SEMA4B			M-SemC	cDNA clone; **group II**	
Sema4C / SEMA4C			M-Sema F (Sem I, M-Sema-F1)	**group I**	
Sema4D / SEMA4D	C-Coll-4	H-CD100	M-Sema G (Sem J, M-Sema-G2)*	**group VI***	
Sema4E / SEMA4E					Sema-Z7
Sema4F / SEMA4F		H-Sema W	M-Sema W	R-Sema W	
Sema4G / SEMA4G					
Sema5A / SEMA5A			M-SemF (M-Sema-F2)		
Sema5B / SEMA5B			M-SemG (M-Sema-G1)		
Sema6A / SEMA6A			M-Sema VIa		
Sema6B / SEMA6B			M-Sema VIb	R-sema Z	
Sema6C / SEMA6C			M-Sema Y	R-sema Y	
Sema7A / SEMA7A		H-Sema K1, H-Sema L	M-Sema L, M-Sema K1*	**group IV***	

The novel nomenclature is indicated in the left column, old names are given to facilitate the orientation in the literature published before the unified nomenclature was adopted. [1] Rodent/Other vertebrates. Invertebrates as well as viral semaphorins are not shown. Groups found in the present study are shown in bold. Asterisks mark the semaphorin member with highest homology to a particular group. cDNA clone stands for partial cDNA sequence found in EST sequence database.

7.2. Semaphorin-Specific Differential Display

7.2.1. The development of a new strategy

Current methods of targeted DD require the replacement of arbitrary primers by targeted primers, specific for any member of a gene family of interest. However, this method is not suitable in all cases. One inherent feature of DD is its bias towards fragments close to the 3' region of mRNA. One of the primers (anchoring) anneals to the polyA tail of mRNA, and the second one should not be far from the first one in order to produce short fragments (ca 200-600 bp) to be separated on a sequencing gel. If the conserved domain of the given gene family (like the Sema domain for semaphorins) is situated far from 3' mRNA end, this approach cannot be used anymore. One possible solution is to replace the oligo(dT) anchoring primer by the arbitrary or the second targeted primer. The function of anchors in oligo(dT) primers is achieved by one or two additional nucleotides, present in 3' end of the primers. This design allows for separation of the total mRNA pool on completely independent groups, according to the anchoring nucleotides, and thus the possibility to cover all possible mRNAs present in the cell is achieved. When using two targeted primers in the display, this feature is not maintained anymore. Therefore, based on the previous method for DD, we developed an improved

strategy for semaphorin-specific DD, designed in two steps (Figure 33). The first step will be an RT-PCR performed with semaphorin-specific degenerate primers under the conditions used to find new semaphorin family members (see §I.1.1). The resulting 900 bp band will thereafter represent a mixture of various semaphorins, and the ratio of each sequence in this mixture will depend on particular experimental conditions. The second step involves a second PCR, an actual DD using sets of arbitrary or semaphorin-specific primers, under conditions similar to those used for the reamplification after DD ((Brenz Verca et al., 1998) and METHODS section), i.e. few steps of amplification at low-stringency conditions, followed by several cycles at high stringency. This strategy, designated "Enriched Differential Display", allows to enrich the template for DD PCR reaction in sequences belonging to the gene family of interest. In addition, it allows us to perform all reactions in agarose gel, thus facilitating the manipulations, decreasing the time needed for the experiments and omitting radioactivity in the PCR.

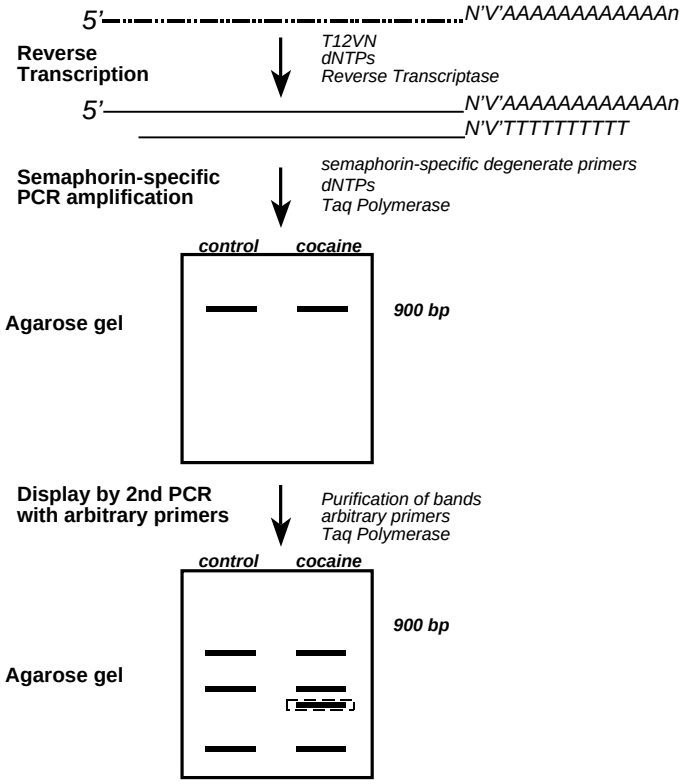


Figure 33. Enriched semaphorin-specific differential display

7.2.2. The design of semaphorin-specific primers

The distribution of all semaphorin-specific primers within the Sema domain is depicted in Figure 34.

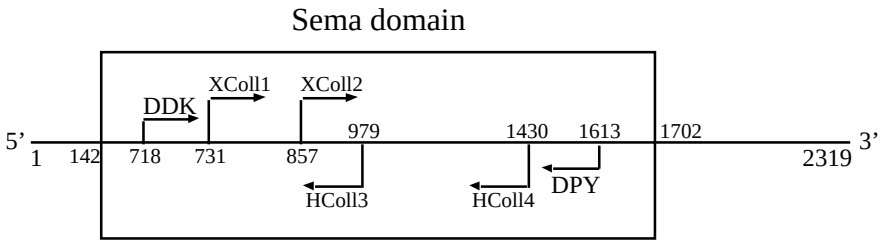


Figure 34. The relative position of Enriched Differential Display semaphorin-specific primers within the Sema domain (this example is for rat Sema3A (R-SemaIII)).

To search for primers for semaphorin-specific Enriched Differential Display, we aligned murine and rat semaphorin sequences from Genbank database together with our cloned new semaphorins from group III (Figure 35). 10 nucleotides were chosen for two forward and two reverse primers in regions of highest

semaphorins homology and designated XColl1 & 2 and HColl3 & 4 respectively. All four primers are located within the part of the Sema domain, amplified by the primers DDK and DPY (Figure 34). We were especially concerned with the binding specificity at the 3' terminus of the primers. The highest specificity was achieved with forward primers (XColl1 and XColl2) as compared to reverse primers (HColl3 and HColl4). The non-semaphorin "tail" of the primers was designed to contain the restriction sites XhoI and HindIII and part of SP6 and T7 promoters to enable the subsequent cloning and reamplification with XSP6 and BT7 primers (Brenz Verca et al., 1998). The sequences and structure of the primers are shown in Table 10.

```
groupIII              -ACTTCTTCTTCCGA-GAG-CGC-CTGCAGA-------G 28
mSemaA                TATTTCTTCTTCCGC-GAGTCCG-CTGTGGA-------A 962
mSemaB                TATTTCTTCTTTGAG-GAGACAG-CCAGCGAG-----TT 826
mSemaC                TACTTCTTCTTCAGC-GAGACGG-GCCAGGAG-----TT 650
mSemaD                TATTTTTTCTTCCGA-GAAAATG-CAATAGG------CG 870
mSemaE                TATTTCTTCTTCAAA-GAAAGAC-TGACTGACAACAATA 897
mSemaF                TACTTCTTCTTCCGA-GAAAACG-CCGTGGAA-----CA 746
mSemaFlike            TACTTCTTCTTCAGT-GAGCGGG-CAGTGGAG-----TA 836
mSemaG                TACTTCTTTTTTACG-GAGGTAT-CCGTGGAG-----TA 785
mSemaH                TACTTTTTCTTTACT-GAGAAGG-CGCTGGAGGCGGAGA 1378
mSemaVIa              TACTTCTTCTTCAGA-GAAATTG-CAGTAGAA-----TA 801
mSemaVIb              TATTTCTTCTTCCGG-GAGATCG-CCATGGAG-----TT 784
mCollapsin1           TATTTTTTCTTCCGA-GAAAATG-CAATAGA------CG 797
rCollapsinIII/1       TATTTTTTCTTCCGA-GAAAATG-CAATAGA------TG 760
XColl1 specific part   ACTTCTTCTT

groupIII              ACAAGTGGAGCGCATTCCTGAAGGCACGGCT 138
mSemaA                ACAAATGGACCACATTTCTGAAGGCGCGGCT 1078
mSemaB                AGAAGTGGACCACCTTCCTCAAAGCCCAGTT 937
mSemaC                AACGCTGGACCTCCTTTCTCAAGGCTCAGCT 761
mSemaD                ATAAATGGACAACATTCCTAAAAGCACGCCT 982
mSemaE                ACAAGTGGACCACATTCTTAAAGGCAAGACT 1003
mSemaF                ACACCTGGACTACCTTCATGAAGGCTCGCCT 854
mSemaFlike            AGAAATGGACGACGTTCCTGAAGGCTCGGTT 947
mSemaG                AAAAGTGGACCTCCTTCCTAAAGGCCAGGCT 896
mSemaH                ACAAGTGGAGCACTTTCCTTAAAGCGCGGCT 1484
mSemaVIa              AGCAGTGGACATCTTTCCTGAAGGCTCGCCT 915
mSemaVIb              AGCAGTGGACTTCCTTCCTGAAGGCCCGGCT 898
mCollapsin1           ATAAATGGACAACATTCCTAAAAGCACGCCT 909
rCollapsinIII/1       ATAAATGGACAACTTTCCTGAAAGCACGCCT 872
XColl2 specific part                  TCCTGAAGGC

groupIII              T-GCTGTCTTCACCTCT-------TCA----GGCTC 264
mSemaA                T-GCTGTCTTCTCCACC-------TCC----AGTGG 1201
mSemaB                C-GCAGTCTTTACCTCCCAGTGG--CAGGTTGGCGG 1060
mSemaC                TCGGGGTCTTTACCTCCCAGTGGCAC-----AGAGG 890
mSemaD                T-GGAGTGTTCACAACA-------TCA----AGCAA 1108
mSemaE                T-GGCATCTTCACCACA-------TCA----AGCTC 1129
mSemaF                T-GGTATCTTCACCACC--------AATGTAAACAG 965
mSemaFlike            C-GGGGTTTTTCAAGCGCGATGGGGCG----ATATG 1071
mSemaG                T-GCGGTCTTCACCCCACAGCTGAACA----ATGTG 1020
mSemaH                T-GGACTGTTTAATACT-------ACC----AGCAA 1610
mSemaVIa              TGGCAACCTTTTTCCACA--------CCTTATAACAG 1035
mSemaVIb              TCGCTGTCTTCTCAACT--------CCTAGCAACAG 1018
mCollapsin1           T-GGAGTGTTCACAACA-------TCA----AGCAA 1035
rCollapsinIII/1       T-GGAGTGTTCACAACA-------TCA----AGCAA 998
HColl3 specific part         TCTTCACCAC

groupIII              ---GAAGAGCTCATG-------TTAGAGGAGGTGGAGG-T 711
mSemaA                TCTGAAGGACTTCTC-------CTGGAAGAGCTGCAGG-T 1648
mSemaB                -----AGTGCTTATCTC-----GTGGAGGAGATTCAGC-T 1450
mSemaC                -----AGTCCACATC-------ATTGAGGAGCTGCAGA-T 1310
mSemaD                CTAGAAGAAGTTCTT-------CTGGAAGAAATGACCG-T 1555
mSemaE                AGTGGGGAACTCATC-------CTGGAGGAGCTGGAAG-T 1576
mSemaF                ----GGCAGCTGTTTG------CTGGAAGAGATTGAGCTT 1377
mSemaFlike            -----GATCCACATG-------GTGGAGGAACTGCAGG-T 1496
mSemaG                -----GGTGCATGTC-------ATCGAGGAGACCCAAC-T 1457
mSemaH                TG-GAGGAAGTCATT-------CTAGAGGAACTTCAAA-T 2063
mSemaVIa              T--GGCAGCCTTTTC-------CTGGAGGAGATGAATG-T 1485
mSemaVIb              AGGGCCCAGCATCTTT------TTGGAGGAGTTTGAGA-C 1468
mCollapsin1           CTAGAAGAAGTTCTT-------CTGGAAGAAATGACCG-T 1482
rCollapsinIII/1       CTAGAAGAAGTTCTT-------CTGGAAGAAATGACAG-T 1445
HColl4 specific part                        TGGAGGAGAT
```

Figure 35. Alignment of 12 murine and 1 rat semaphorin sequences from the Genbank with the group III of our newly cloned semaphorins . Only the fragments of entire alignment are shown containing the sequences chosen for the Enriched Differential Display primers. Consensus sequences used for primer formulation are shown in bold below the alignment.

Table 10. Semaphorin-specific differential display primer sequences.

Forward primers (X stands for XhoI site): XColl1: ACACTATAG CTCGAG **ACTTCTTCTT** XColl2: ACACTATAG CTCGAG **TCCTGAAGGC**
Reverse primers (H stands for HindIII site): HColl3: CACTATAGGG AAGCTT **GTGGTGAAGA** HColl4: CACTATAGGG AAGCTT **ATCTCCTCCA**

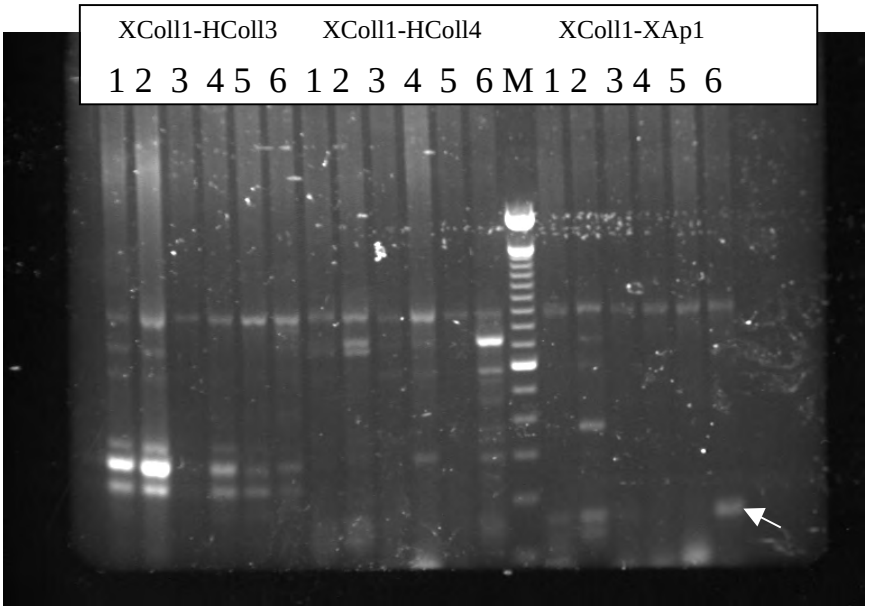
Enriched Differential Display primer sequences chosen based on the alignment of the nucleotide sequences of 14 rodent semaphorins. Semaphorin-specific sequences are in bold, restriction enzyme XhoI and HindIII sites are underlined, the rest of the sequences corresponds to part of T7 or SP6 RNA polymerase promoters.

7.2.3. Preliminary results

Preliminary experiments of Enriched Differential Display are shown on Figure 36. It can be observed that the pattern of display is specific for a particular primer pair, and some net differences can be seen between saline- and cocaine- treated brains as well as between different brain regions, as shown by arrows on the figure. However the background varies in intensity between several spots. This can be due to problems of standardization (discussed later, §III.7.2.4). Only the differences clearly distinct from the otherwise homogeneous background should be chosen for further analysis. Some differences are specific for only one particular brain region (one candidate labeled in XColl1-XAp1 was only obvious in the NAcc). Some other fragments have shown parallel changes in two regions simultaneously. For example, the XColl1-XAp7 combinations yielded three fragments, down-regulated in both the LStr and the Hipp (the two brightest ones are indicated by arrows on Figure 36). The combinations of two semaphorin-specific primers yielded less differences than combinations of one targeted and one arbitrary primer.

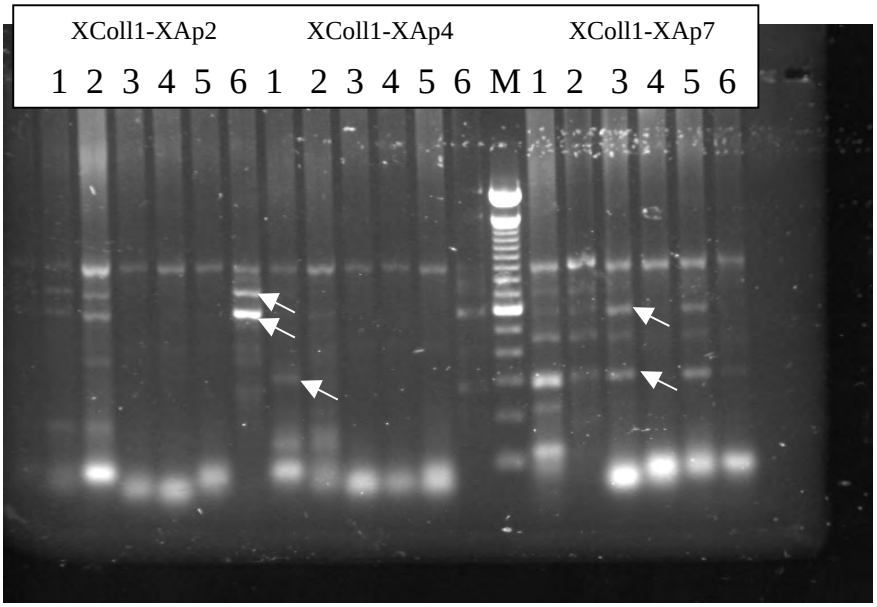
A very detailed analysis of semaphorin-specific Enriched DD, based on these ideas and methodology and on these preliminary data, has been the focus of Dr. David AJ Widmer's thesis, elaborated in our laboratory and submitted and accepted at the Rutgers University (August 2000). 58 fragments have been found to be up- or down-regulated after acute cocaine administration in eleven differential displays with different combinations of primers. 46 of the differentially displayed fragments were sequenced. Several fragments represented different parts of the same sequences. Of these, six were members of the semaphorin axon guidance family, eleven had strong homology to other molecules, and another thirteen were novel gene sequences with no clear homology to any Genbank records. All semaphorins were already identified from the first experiment shown on Figure 36. However, not all changes could be reproduced in duplicate/triplicate experiments and need to be confirmed by other methods, such as quantitative RT-PCR. The low reproducibility of the method as compared to the standard DD, could be due to potential problems discussed later (III.7.2.4. The more consistent changes in semaphorin expression included Sema3D and Sema4B up-regulation in the NAcc, Sema4D down-regulation in the NAcc, and Sema5A over-expression in the Hipp. The potential implications of these findings in drug addiction studies is dealt with in the DISCUSSION section.

Figure 36. Enriched Differential Display on mRNAs prepared from different parts of cocaine- or saline-treated brains with several combinations of arbitrary and semaphorin-specific primers



1: sAcc
2: cAcc
3: sLStr
4: cLStr
5: sH
6: cH

s: saline
c: cocaine
Acc: Nucleus Accumbens
LStr: Lateral Striatum



7.2.4. Potential drawbacks of the new method

Two potential problems can be stated in regard to this method:

1. The identical starting amount of cDNA from the sample and from the control, used for the first PCR in the Enriched Differential Display, is an absolute prerequisite for the success of the method. Any variation in the initial cDNA material will be exponentially amplified during the 2nd PCR reaction, i.e. the actual DD, and the risk of false positives will substantially increase. All reverse transcription reactions are performed with the same amount of RNA, but the efficiency of reaction as well as percent of cDNA recovered after EtOH precipitation step may differ from tube to tube and it is impossible to directly measure the cDNA concentration by OD readings or densitometric quantification on gel. Therefore, the possible solution to this problem will be a careful quantification of the cDNA template by quantitative PCR technique with GAPDH or another endogenous control.
2. The 1st PCR step should be as specific as possible. In case of multiple PCR products at this step the bands should be purified on gel, which again implies variability in the starting material for the 2nd PCR reaction. The sources of such variability are: products of variable sizes after the 1st PCR (from 870 to 910 bp approx.) or variation in the recovery during gel extraction or elution procedures. Therefore an aliquot of the 1st PCR reaction is used directly for the 2nd PCR. The optimal dilution of this aliquot should be determined in order to achieve the most informative screening.
   The required specificity of the 1st PCR could be accomplished by a touch-down PCR program. The annealing temperature is progressively diminished at every 5 cycles of PCR. At the last cycles, when the annealing temperature is low and thus permissive for non-specific binding, the concentration of primers available for annealing is low, and a possible contamination remains insignificant.

Another issue in the Enriched Differential Display resides in choosing either arbitrary or semaphorin-specific primers for the 2nd PCR. We have opted for the second possibility, which may maintain semaphorin-specificity allover the procedure, avoiding the amplification of non-semaphorin sequences as contaminants of the cDNA. Already during the RT-PCR leading to new semaphorin members about 20% of the sequences were non-semaphorins, due to the limited specificity of the primers used in the reverse transcription reaction. Semaphorin-specific primers in the 2nd PCR from both sides will yield fragments of the same length, so an ideal compromise resides in using one semaphorin-specific primer and a second arbitrary primer.

IV. DISCUSSION

In this work the differential display study was undertaken to find out the genes differentially expressed in response to cocaine treatment in the striatum, the hippocampus and the tegmentum of rats. The screening resulted in 47 differentially expressed fragments, ten of which have homology with mRNAs of known proteins. In addition, CART up-regulation in the nucleus accumbens (NAcc) was shown. These observations add new data to previous knowledge about gene expression upon cocaine treatment, in particular in the protocol of early drug withdrawal applied in this study, which previously resulted in finding of ephrin-B2 up-regulation (Yue et al., 1999). Two of the identified proteins, CD81 and γ-synuclein, up-regulated in the NAcc and tegmentum respectively, have been studied in more detail, and two papers extensively describe these studies. In this section we will introduce the eight other candidates and discuss their possible involvement in drug addiction.

1. *Sodium bicarbonate transporter*

We have found up-regulation in the hippocampus and down-regulation in the entire striatum of the electrogenic $Na^+/HCO3^-$ cotransporter (NBC). Bicarbonate transporters are the principal regulators of pH in animal cells, and are expressed in the stomach, pancreas, intestine, kidney, reproductive and central nervous systems. The functional family of $HCO3^-$ transporters includes Cl^- -$HCO3^-$ exchangers, three NBC isoforms, a $K^+/HCO3^-$ cotransporter, and a Na^+-driven Cl^- -$HCO3^-$ exchanger (Romero et al., 1997). NBC is responsible for the transport of $HCO3^-$ across the basolateral membrane in kidney, where approximately 80% of the filtered load of $HCO3^-$ is reabsorbed in the proximal tubule via a process of active acid secretion. Cases of differential regulation of NBC have been reported previously. For example, NBC-1 is up-regulated in metabolic acidosis and K^+ depletion and in response to glucocorticoid excess and is down-regulated in response to $HCO3^-$ loading or alkalosis (Soleimani and Burnham, 2000). NBC is expressed in neurons, and especially in glial cells in the brain and plays an important role in the regulation of both intracellular and extracellular pH. NBC is responsible for astrocyte vulnerability to acidic injury, the factor contributing to stroke after cerebral ischemia. The NBC expression profile in brains suggests that this transporter is critical in later stages of brain development, during the time of generation and maturation of astrocytes (Giffard et al., 2000). Interestingly, dopamine (DA) inhibits the NBC in renal proximal tubules, the effect being mediated by D1 receptor signaling (Kunimi et al., 2000). Cocaine also activates dopaminergic (DAergic) signaling via this type of receptor, and thus may regulate the transporter expression in this way. On the other hand, this could also be a response of glial cells, the major cells expressing the NBC, to cocaine action. In both cases the modified NBC activity would necessarily influence both the extracellular and intracellular pH of the surrounding neurons, which in turn would affect their excitability. This could be a new compensatory mechanism involved in cellular response to drug action.

2. *Olfactomedin-related protein or pancortin*

We have found increased expression in the striatum of the olfactomedin-related protein, or pancortin, upon cocaine treatment. Olfactomedin is a 57-kDa glycosylated extracellular matrix (ECM) protein of unknown function originally identified in the amphibian olfactory neuroepithelium. The olfactomedin homologues have been identified in fish, frog, rat, mouse and human. The high evolutionary conservation of its primary structure supports the notion that olfactomedin has an important function in the mammalian nervous system (Karavanich and Anholt, 1998). Olfactomedin contains cysteines which form disulfide-linked polymers that constitute the primary architecture of the olfactory ECM. By analogy to other ECM proteins of the nervous system, olfactomedin may influence the maintenance, growth, or differentiation of chemosensory cilia of olfactory neurons (Yokoe and Anholt, 1993). The massive production of olfactomedin and its striking deposition at the chemosensory surface of the olfactory neuroepithelium suggest a more specific role for this protein in chemoreception (Snyder et al., 1991).
More recently, two independent groups have cloned four olfactomedin-related proteins (in rat) or pancortins (in mice, called pancortins for their massive expression in the cerebral cortex) (Danielson et al., 1994; Nagano et al., 1998). All members of this new family share a common central region (Figure 37) and variable N- and C-terminals.

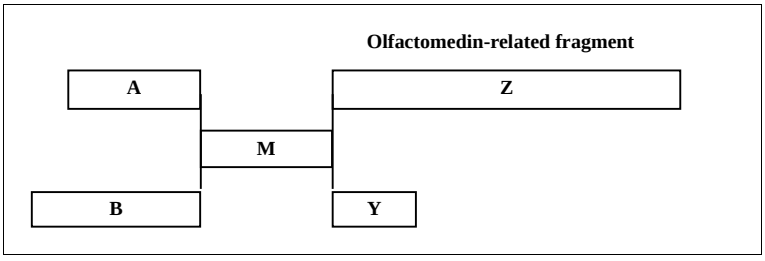


Figure 37. Schematic representation of how the five pancortin cDNA blocks are linked to generate the four transcripts AMY, AMZ, BMY, and BMZ. Names are given according to the nomenclature proposed by the group who first identified the proteins [Danielson, 1994 #161]

.

The distinct mRNAs are generated from a single gene by a tissue-specific and developmentally regulated choice of two alternative 5' ends (A or B). Alternative splicing further generates RNAs with two different 3' ends (Y or Z) of roughly equimolar concentrations. The homology to olfactomedin is found in the Z region of long forms, which also have the carboxy terminal sequence KDEL, a functional endoplasmic reticulum anchor sequence in *Plasmodium falciparum* (Danielson et al., 1994; Munro and Pelham, 1987). However, the homology to olfactomedin as well as the presence of putative signal peptide sequences indicate that these proteins are secreted. Later studies suggested the possibility for A region-containing pancortins to be extracellular proteins, conversely to B-pancortins (Nagano et al., 2000). The pancortins are mainly expressed in the olfactory bulb, the cerebral cortex and the hippocampus. However, the distribution of particular subunits in the brain, studied by *in situ* hybridization in rat or mouse brain, remains a controversial issue (Danielson et al., 1994; Nagano et al., 1998).

It is difficult to propose a role for olfactomedin-related proteins in cocaine action, because the function of neither pancortins nor olfactomedin is clear. We know, however, that the mRNAs of pancortins are unstable, and therefore the level of protein should be regulated transcriptionally. Pancortin has also been identified by differential display, an RNA-biased method, as one of the genes expressed in the pons during the neuronal circuit formation period (Nagano et al., 1998). Different pancortins are differentially regulated during development. This suggests a role in differentiation and/or survival of neurons, probably by furnishing them an appropriate extracellular environment. Neuroplasticity changes in cocaine-treated brain need also a specific extracellular environment, and the expression of ECM proteins might be regulated correspondingly. It is known that the migration of DAergic cells in the embryonic mesencephalon depends upon ECM cues like tenascin (Kawano et al., 1995), and the secretion of DA is facilitated in cells grown on ECM substrate (Bethea, 1987). Moreover, tenascin-knockout mouse display a paradoxical behavioral response to apomorphine (Fukamauchi et al., 1997), favoring the idea of a role of ECM molecules in DAergic system both in developing and adult brain.

3. *Metabolic enzymes*

We have found four fragments, two of them encoded by the mitochondrial genome, homologous to metabolic enzymes:

1) Pyruvate dehydrogenase E1 alpha form 1 subunit, down-regulated in the tegmentum;
2) NADH dehydrogenase subunit 6 (mitochondrial genome), up-regulated in the tegmentum;
3) Cytochrome c oxidase (Cox), subunit III (mitochondrial genome), down-regulated in the tegmentum;
4) Cox, subunit VIIc, up-regulated in the hippocampus.

The pyruvate dehydrogenase complex is an assembly of three enzymes, catalyzing the oxidative decarboxylation of pyruvate to acetyl CoA. The E1 component of the complex is the pyruvate decarboxylase subunit itself. NADH dehydrogenase and Cox belong to different mitochondrial membrane complexes involved in oxidative phosphorylation (Wallace, 1992). The electrons of NADH enter the respiratory chain at NADH dehydrogenase, a large enzyme consisting of about 25 polypeptide chains. Cox is the terminal enzyme in the respiratory chain and is located in the inner membrane of mitochondria. It catalyses the reduction of dioxygen to water and pumps an additional proton across the membrane. The resulting electro-chemical gradient is used in the synthesis of ATP (Hofacker, 1999). Cox of mammals is found in a dimeric state, with each monomer containing thirteen subunits. Genes for subunits I, II, and III, which represent the catalytic core of the enzyme, are encoded by mitochondrial DNA. The remaining ten, smaller regulatory subunits, are encoded by nuclear DNA (Lenka et al., 1998). Some Alzheimer's patients have a missense mutation in the mitochondrial genes for Cox. The mutation appears to cause a decrease in the enzyme activity and a corresponding increase of reactive oxygen species (ROS) which leads to cell death. Cox also affects aggregation of beta-amyloid peptide in Alzheimer's disease's associated plaques (Scott).

The regulation of NADH dehydrogenase subunit 6 in the mesoaccumbal DAergic system by cocaine has been reported previously (Couceyro et al., 1997). These changes correlate with other known cocaine effect on metabolism, i.e. alteration of 2-deoxyglucose uptake (Table 11) (London et al., 1990). Both 2-deoxyglucose uptake and NADH dehydrogenase expression are diminished by cocaine self-administration in the NAcc. At the same time, enzyme expression is up-regulated in the tegmentum, which may account for the adaptive response of the DAergic neurons.

Methamphetamine administration produces selective damage to DAergic nerve terminals, which is due to the release of DA from synaptic vesicles within the terminals, allowing the generation of ROS via DA metabolism (Wei et al., 1997). The possible neurotoxicity of cocaine is still debated, but its hepatotoxic effect is associated with increased mitochondrial generation of ROS (Boess et al., 2000), which preferentially damage the pyruvate dehydrogenase and Cox complexes (Gibson et al., 1998). This would account for their decreased expression in the tegmentum following cocaine treatment (Table 11). It is more difficult however to

explain the over-expression of several Cox subunits in the hippocampus and the striatum (Table 1). Again this could be an adaptive, neuroprotective response to over-stimulation of neurons. The down-regulation of Cox subunit VIIc has been associated with apoptotic processes in macrophages (Ragno et al., 1998). Otherwise, the discrepancies in metabolic enzyme responses on drug treatment can be explained by differences in the protocols used in different studies (Table 11). For example, both acute high dose and chronic metamphetamine treatments resulted in decrease in Cox in the striatum and substantia nigra (Burrows et al., 2000; Prince et al., 1997), as opposed to the data mentioned in the Table 11.

Table 11. Changes in brain metabolism induced by cocaine treatment

Enzyme / brain region	Hipp	NAcc	LStr	VTA[5]
Pyruvate dehydrogenase				⇓[1]
NADH dehydrogenase	⇓[2]	⇓[2]	no changes[2]	⇑[1,2]
Cox subunit I		⇑[3]	⇑[3]	no changes[3]
subunit III		⇑[3]		⇓[1], no changes[3]
subunit IV		⇑[3]		no changes[3]
subunit VII	⇑[1]			
2-DG uptake		⇓[4]		

Changes in brain metabolism induced by cocaine treatment are presented here as assessed by measures of metabolic enzymes expression or 2-deoxyglucose (2-DG) uptake. ⇑ and ⇓ indicate increase or decrease of corresponding parameters, respectively. [1]differential expression found in the present study; [2]NADH dehydrogenase expression upon cocaine self-administration (Couceyro et al., 1997); [3]cytochrome c oxidase (Cox) expression upon 2 weeks passive administration of cocaine (Walker and Sevarino, 1995); [4]2-DG uptake upon cocaine self-administration (Hammer et al., 1993); [5]in the present study the entire tegmentum was analyzed. Hipp: hippocampus, NAcc: nucleus accumbens, LStr: lateral striatum, VTA: ventral tegmental area.

It is clear from previous as well as from the present study that cocaine interferes with brain metabolism. It is still unknown whether these changes represent a general effect of drug treatment, or more specific phenomenon related to drug reinforcing action. In addition, the particular involvement of Cox in this process may correlate with cocaine effects on increased production of ROS, impairment of mitochondrial function and apoptosis.

4. *Mss4*

A signal transduction molecule, which was found in the present study to be over-expressed in the hippocampus upon cocaine treatment, is the guanine-nucleotide-releasing factor (GRF) mss4. The involvement of the hippocampus in drug addiction has not been discussed so far, however there is a substantial amount of data illustrating this phenomenon. For example, cocaine-exposed offspring exhibit consistent hippocampal abnormalities as adults (Baraban et al., 1999). Intrahippocampal injections of morphine is rewarding. The most robust reward appears to be in CA3 region, an area rich in opiate receptors and endogenous opiate peptides. Mu-type receptors, linked to postsynaptic Gi proteins, seem particularly important for this effect (Bardo, 1998). Chronic administration of cocaine was associated with increases in nitric oxide synthase activity in the hippocampus (Bhargava and Kumar, 1997). In addition, repeated cocaine administration down-regulated glucocorticoid receptors in the rat brain cortex and in the hippocampus (Budziszewska et al., 1996). Cocaine-induced activity increases in many regions of the brain, including the striatum, the basal forebrain, the hippocampus and the ventral tegmentum as measured by functional MRI (Breiter et al., 1997). The effects of cocaine in the hippocampus may be related either to general effects of the drug on brain function or to more specific interference with hippocampal activity. The hippocampus is a structure which plays a major role in the processes of memory. Cocaine also interferes with memory processing in the brain by influencing both the reinforcement and associative types of learning. Not surprisingly, exposure to cocaine self-administration environment enhanced Fos expression in the hippocampal CA1 region, dentate gyrus and NAcc, even without the associated injection of cocaine (Neisewander et al., 2000). The mechanism of cocaine action may be due to the sustained enhancement of AMPA receptor- and NMDA receptor-mediated currents induced by DA D1/D5 receptor activation in the hippocampus (Yang, 2000). In addition, DA may facilitate the acetylcholine release in the hippocampus, thus regulating hippocampal cognitive processes (Imperato et al., 1993). DA agonists have strain-specific action on hippocampal acetylcholine release in mice and on memory consolidation (Imperato et al., 1996). Cocaine is also able to block the induction of LTP at the excitatory synapses in the CA1 region of the hippocampus independently of NMDA receptors or

channels (Smith et al., 1993). Both repeated and acute cocaine administration decreased the Goα mRNA level in the CA3 region and increased it in the CA1 at various time points. Furthermore, in all hippocampal regions, acute, but not chronic, cocaine administration increased the Gsα mRNA level at 24 h, whereas chronic treatment significantly decreased that level 48 h after the last dose (Przewlocka et al., 1994). This last finding is of particular relevance here, because changes in synaptic plasticity, ultimately leading to changes in long-term memory, involve the regulation of G-proteins. The stimuli that evoke plasticity induce the mRNA encoding for a member of the regulator of G-protein signaling (RGS) family, RGS2, in neurons of the hippocampus, the cortex, and the striatum (Ingi et al., 1998). Ras subfamily of small G-proteins have been implicated in synaptic function and region-specific learning and memory functions (Kawasaki et al., 1998). Mice that lack the Ras-GRF, have severely impaired LTP in the amygdala and a corresponding deficit in long-term memory for aversive events (Orban et al., 1999). Mss4 is a GRF for the Sec4/Ypt1/Rab family of small G-proteins involved in the regulation of intracellular vesicular transport (Muller-Pillasch et al., 1997). It interact with Rab3a, a small G-protein mainly expressed in neural and endocrine cells which might regulate exocytosis (Darchen et al., 1995). The Rab3a plays a role specifically in the recruitment of synaptic vesicles for exocytosis during repetitive stimulation (Geppert et al., 1994). Mss4 also regulates rab8 which plays a role in membrane trafficking during neuronal process outgrowth (Huber et al., 1995). Finally, mss4 binds to cytoplasmic domains of α-integrin, and may therefore provide the link between ECM, integrins and intracellular messenger cascades (Wixler et al., 1999).

The up-regulation of mss4 in the hippocampus might therefore reflect the implication of cocaine in memory processes. The multiple interaction pattern of mss4 with other proteins suggest different mechanisms for this phenomenon, including the release of neurotransmitter, neuronal process outgrowth and integrin-dependent cellular events.

5. *Ecto-apyrase*

Nucleotides such as ATP, ADP, UTP or the diadenosine polyphosphates are extracellular signaling substances in the brain and in other tissues. Enzymes located on the cell surface catalyze the hydrolysis of these compounds and thus limit their spatio-temporal activity. In nervous tissue nucleoside 5'-triphosphates are hydrolyzed by ecto-ATP-diphosphohydrolase (ecto-apyrase) and possibly also by ecto-nucleoside triphosphatase and ecto-nucleoside diphosphatase (Zimmermann, 1996). We have found the ecto-apyrase expression to be increased in the tegmentum upon cocaine treatment.

Cocaine exerts its action in the tegmentum, essentially in the VTA, both directly and indirectly, through the NAcc neurons. The increased synaptic levels of DA in the NAcc activates a long-loop negative feedback pathway to the VTA involving both D1 and D2 postsynaptic receptors (Rahman and McBride, 2000). However the inhibitory effects of cocaine on VTA neurons are relatively weak and may represent a poor compensatory response to enhanced DA neurotransmission within the NAcc. This may explain the extremely potent rewarding effects of cocaine (Einhorn et al., 1988). In addition, the D1 receptors of the VTA are selectively involved in behavioral sensitization induced by intra-VTA injections of D-amphetamine (Bjijou et al., 1996). The direct effect of cocaine in the VTA includes blocking the reuptake of DA released from the dendrites of the A10 DA neurons, thus prolonging the actions of DA at D2 autoreceptors (Cameron and Williams, 1994). The uptake of DA as well as other catecholamines into synaptosomes is potentiated by ATP in a dose-dependent manner (Cao et al., 1990). The similar effect could happen *in vivo* as a compensatory reaction to inhibited DA reuptake, and therefore explain the up-regulation of ecto-apyrase, needed to metabolize the excess of extracellular ATP. It was shown that DAergic principal neurons of the VTA do not possess somatic P2 purinoceptors present on peripheral and central noradrenergic neurons (Poelchen et al., 1998). However, the effect of ATP on DAergic neurons was examined in the absence of the drug. Cocaine treatment could therefore activate the ATP signaling in the VTA, and our finding of all-or-none expression of ecto-apyrase strongly favors this hypothesis.

6. *Semaphorins*

In the present study we found one new vertebrate member of the semaphorin family, with a closest homology to avian SEMA3D, and five novel rat orthologues of class 3, 4 and 7 semaphorins. In addition the method of Enriched Differential Display was developed in order to perform the screening for differentially expressed members of the semaphorin family. The subsequent application of this method in our group (Widmer, 2000) resulted in the finding of semaphorin differential regulation in various brain regions upon cocaine treatment. Namely, Sema3D and Sema4B were up-regulated, whereas Sema4D was down-regulated in the NAcc, and Sema5A was over-expressed in the hippocampus. Sema3D is a guidance cue for the midline crossing of axons and for the elongation and synapsing of retinal axons in the avian optic tectum (Luo et al., 1995). Sema4B and Sema4D are both guiding molecules, one for the olfactory neurons invading the olfactory bulb, and the other for the immature thymocytes to the medulla of the thymus respectively, during development (Tamagnone et al., 1999; Williams-Hogarth et al., 2000). Finally, Sema5A is a transmembrane protein, possibly acting as a permissive cue for growing axons (Adams et al., 1996). Its cytoplasmic location was mapped to the deletion related to Cri-du-Chat syndrome (Simmons et al., 1998). The involvement of multiple semaphorins in cocaine action suggests the activation of a complex program of synaptic rearrangements induced by the drug, which may recapitulate the plastic changes occurring during development in terms of mechanisms and of the molecular players involved.

In conclusion, we found several genes, differentially regulated in several regions of the rat brain in response to cocaine treatment. The proteins for which the mRNAs found are coding, display a high range of functional characteristics, illustrating the complexity and the variability of neuroplastic changes induced in the brain by psychostimulant treatment. The functional significance of the proteins suggests that important metabolic and neuroplastic changes occur in the reward- and memory-related brain centers following drug action. Four metabolic enzymes were found to be differentially expressed, in line with previous findings concerning brain metabolism in cocaine addiction. Other proteins show an involvement of synaptic plasticity. The putative ECM protein pancortin, as well as mss4 and CD81, two integrin-binding proteins, could affect the neuroplasticity by providing the necessary extracellular environment. The transmembrane proteins NBC and ecto-apyrase could affect the neuronal excitability by changing the conductance properties of neuronal membrane or more directly, by regulating the level of extracellular messengers. Finally, CART, synucleins and axon guidance molecules of the Eph-ephrin or the semaphorin families, could directly influence the morphology and synaptic rearrangement of DAergic cells. The additional search for other molecules expressed in the same time point, as well as a detailed study of the functional significance of already identified molecules will further clear up the exact picture of molecular and cellular events involved in early stages of drug addiction.

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Annex I

Modification of Primer Design Facilitates the Use of Differential Display

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The method of differential display developed by Liang and Pardee (10) has gained a large audience and is now the method of choice to quickly identify and isolate differentially expressed genes in several experimental systems. The method is powerful because of its inherent simplicity, and it allows the simultaneous comparison of up- and down-regulated genes with small amounts of raw material. Nevertheless, several vulnerable spots have been identified in recent years. Most criticisms concerned false positives generated by the polymerase chain reaction (PCR) step (9). In addition, the identification of differentially expressed genes remains tedious and limited by the fact that the amplified cDNA fragment generally corresponds to the trailer region of mRNA. Additional difficulties are: *(i)* the contamination of bands recovered from the display gel by heterogeneous sequences (1) and *(ii)* the strong bias in favor of the abundant mRNAs (2). For these reasons, many authors have attempted to improve the method, either through different primer designs (19,20) or by adapting techniques for faster confirmation of differential gene expression (13,19) and developing methodologies for easier identification of true positives (3,11). The use of longer primers instead of the original 10-mers has been proposed to increase the specificity and reproducibility of the method (4,10,20) by favoring more specific hybridization in the PCR. This technique integrates the principles of differential display with those of RNA fingerprinting by arbitrarily primed PCR (18). PCR is performed in two steps: *(i)* a few initial low-stringency cycles allowing minor mispairing between the arbitrary primer and the cDNA template, followed by *(ii)* several high-stringency PCR cycles favoring perfect matches with the anchored primers.

Inspired by these improvements, we developed a novel primer strategy aimed at facilitating re-amplification, analysis and cloning of the cDNA fragments (Figure 1A) in which the main feature is the use of a universal re-amplification primer set. The primers are modified with the addition of a constant region at either end of the product; this is obtained by extending the 5′ terminus of the oligo(dT) primers (DLP) and the arbitrary primer (XAp) (Life Technologies, Basel, Switzerland) with about one half of the T7 promoter (Figure 1A, f) and one half of the SP6 RNA polymerase promoter (Figure 1A, a), respectively. Universal re-amplification primers harboring the complete cognate

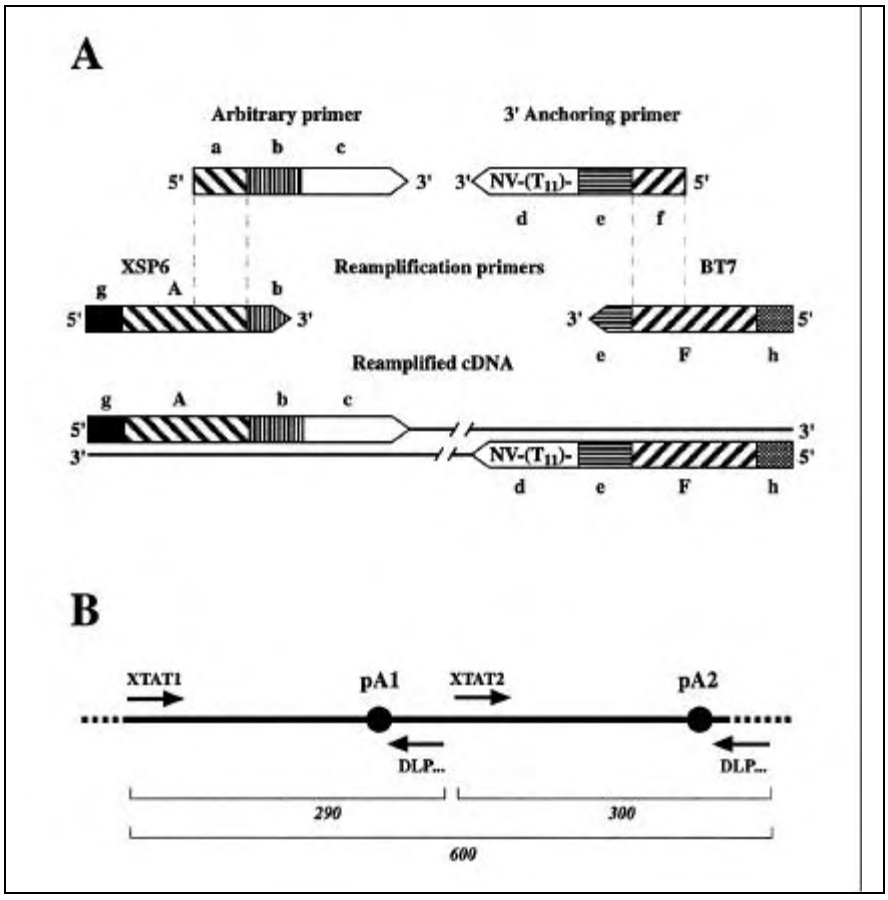

Figure 1. Modified primers for differential display. (A) Modified primer strategy for differential display. Top: arbitrary primers (25-mers) consist of a constant portion composed of one half of the SP6 RNA polymerase promoter (a) in the 5′ region, an *Xho*I restriction site (b) in the middle followed by an arbitrary decamer sequence (c) in the 3′ region. The 3′ anchor primers (27-mers) bear a constant portion composed of a *Hin*dIII restriction site (e) and one half of the T7 RNA polymerase promoter (f), both located upstream of the anchoring oligo(dT) sequence (d). Middle: universal re-amplification primers (29- and 30-mers) contain the entire phage polymerase promoter: SP6 for the 5′ re-amplification primer (A) and T7 for the 3′ re-amplification primer (F), preceded by an additional restriction site: *Xba*I (g) and *Bam*HI (h), respectively. Re-amplification primers hybridize to the constant portion of the cognate anchor primers. Bottom: the final flanking sequences after differential display and cDNA re-amplification contain two RNA polymerase promoters for preparing sense and antisense riboprobes and four restriction sites for further cloning. The sequences of the primers are the following. Oligo(dT) primer sets: DLP1, 5′-CAC TAT AGG GAA GC(T)$_{11}$VG-3′; DLP1 to DLP4 primer sets have a wobble in the penultimate position (V = G, C or A) and differ in their last nucleotide, which is G, C, A and T for DLP1, DLP2, DLP3 and DLP4, respectively; arbitrary primers: XAp1, 5′-ACA CTA TAG CTC GAG *AGG TGA CCG T*-3′; XAp1 to XAp20 differ in their ten last nucleotides (shown in italics), which correspond to the decamer arbitrary sequences described by Bauer et al. (1); re-amplification primers: BT7, 5′-GCG GAT CCT AAT ACG ACT CAC TAT AGG GAA-3′ and XSP6, 5′-GCT CTA GAT TTA GGT GAC ACT ATA GCT CG-3′. Restriction sites are underlined. (B) TAT gene 3′ region with location of polyadenylation signals and priming sites for targeted primers. The rat TAT gene has two polyadenylation signals (pA1, pA2) (5). The arbitrary portion of primers XTAT1 and XTAT2 was chosen to specifically prime at the indicated locations (XTAT1: 5′-ACA CTA TAG CTC GAG *CTC CCA CCC A*-3′; XTAT2: 5′-ACA CTA TAG CTC GAG *AGG AGG ACA C*-3′). The approximative length of the expected cDNAs is indicated.

Table 1. cDNA Fragments Generated from Arbitrary Primers XTAT1 and XTAT2 and Corresponding to mRNA Whose Expression was Modified after Dexamethasone (Dex) Treatment

Lane	Fragment Size (in bp)	Arbitrary Primer	DLP Primer or Set	Identification	Effect Exerted by Dex
6	600	XTAT1	DLP3	tyrosine aminotransferase	+
8	600	XTAT1	DLP4	uncharacterized	0
10	280	XTAT1	DLPAG	tyrosine aminotransferase	+
10	580	XTAT1	DLPAG	uncharacterized	0
10	900	XTAT1	DLPAG	α_2-macroglobulin	+
12	270	XTAT1	DLPAT	metallothionein I	+
12	600	XTAT1	DLPAT	tyrosine aminotransferase	+
14	310	XTAT2	DLP1	tyrosine aminotransferase	+
16	300	XTAT2	DLP2	tyrosine aminotransferase	+
18	270	XTAT2	DLP3	uncharacterized	+
18	290	XTAT2	DLP3	tyrosine aminotransferase	+
18	300	XTAT2	DLP3	tyrosine aminotransferase	+
20	300	XTAT2	DLP4	metallothionein I	+
22	310	XTAT2	DLPAG	tyrosine aminotransferase	+
24	250	XTAT2	DLPAT	metallothionein I	+
24	300	XTAT2	DLPAT	tyrosine aminotransferase	+

Effect exerted by dexamethasone may be up-regulation (+), down-regulation (-) or no change (0). Lane numbers given in the first column are according to Figure 2A. Non-changing bands (0) were included as negative controls.

promoter sequences (Figure 1A, A and F) will prime within these constant regions and hence add a functional RNA polymerase promoter into the re-amplified cDNA. Thus, the terminal sequences of re-amplified cDNAs will contain a T7 RNA polymerase promoter (Figure 1A, F) allowing direct generation of antisense riboprobes for hybridization techniques such as Northern blot, in situ hybridization or ribonuclease protection analysis and an SP6 promoter (Figure 1A, A) for sense RNA synthesis (e.g., to be used as control). This configuration allows variation of the arbitrary and oligo(dT) portions in the initial PCR primers while maintaining the same universal re-amplification primer pair. Direct sequencing of re-amplified cDNAs without previous cloning is possible, independently of the initial primer combination, by using the same particular primer (XSP6).

Whenever cloning is necessary, two restriction sites are located within the arbitrary and oligo(dT) primers (Figure 1A, b and e, respectively). Two additional restriction sites (Figure 1A, g and h) are added by the universal re-amplification primers and provide increased flexibility for cloning purposes.

We illustrate the performance of the modified primers using FTO-2B rat hepatoma cells (7). In this cell line, the tyrosine aminotransferase (TAT) gene expression is induced by glucocorticoids (15,16), providing us with a valuable positive control for mRNA display. Therefore, in addition to the normal arbitrary primers, we designed two others (XTAT1 and XTAT2) with the arbitrary portions substituted by sequences specifically priming at defined locations in the TAT gene (Figure 1B). The rat TAT gene has two polyadenylation signals (pA1 and pA2, in which pA2 is more frequently used) generating RNAs of different lengths (Figure 1B). Because the distance between the exact polyadenylate tail addition point and the polyadenylation signal may vary (17), in a differential display reverse transcription (DDRT)-PCR experiment the TAT gene signal is not expected to be found in any particular 3′ primer set.

The results of differential display performed with XTAT1 and XTAT2 primers are shown in Figure 2A (experimental conditions given in the legend).

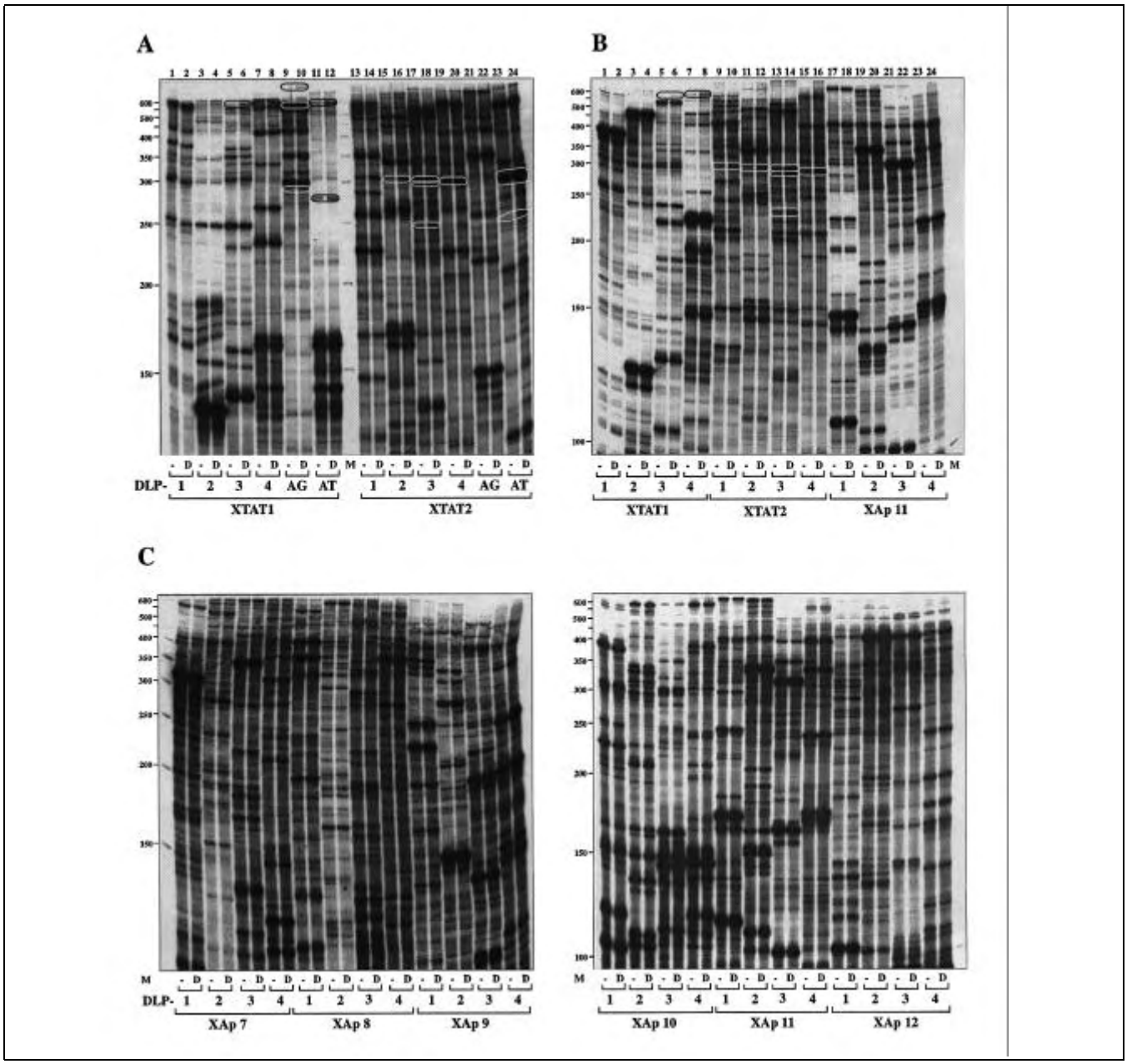

Figure 2. Differential display of mRNA from FTO-2B rat hepatoma cells after dexamethasone treatment. Cells were cultured in Dulbecco's modified Eagle medium (DMEM) with 10% fetal calf serum and treated with 0.5 µM dexamethasone (Sigma Chemie, Buchs, Switzerland). Total RNA was extracted 48 h after addition of dexamethasone. Control RNA was extracted from untreated cells grown under identical conditions. After DNase I treatment (Sigma Chemie), 2 µg total RNA were reverse-transcribed with the oligo(dT) primer sets DLP1–DLP4 according to Liang and Pardee (10). Subsequent PCR was performed with the same sets of oligo(dT) primers in addition to arbitrary primers XAp1–XAp20 or targeted primers XTAT1 and XTAT2. In some cases, primer combinations used DLPAG and DLPAT (particular components of the DLP1 and DLP4 sets, respectively) as oligo(dT) primers, as indicated. PCR was performed as follows: five cycles at low stringency (94°C for 60 s, 40°C for 120 s, 72°C for 60 s) followed by 35 cycles at high stringency (94°C for 45 s, 56°C for 60 s, 72°C for 60 s) on a Biometra TRIO™ thermal cycler (Biolabo, Châtel-St.-Denis, Switzerland). A typical PCR was carried out in a 20-µL final volume with the following final reagent concentrations: 2 µM dNTPs, 1 µM 3′ anchor primer, 1 µM arbitrary primer and 1 mM $MgCl_2$. cDNA (1/50 of that obtained from an RT), 1–2 µCi [α-^{32}P]dATP at 3000 Ci/mmol (Hartmann Analytic, Braunschweig, Germany) and 1.5 U *Taq* DNA polymerase (Life Technologies, Gaithersburg, MD, USA) were used for each PCR. Reaction products were resolved on 6% denaturing sequencing gels that were dried and exposed to Fuji X-ray film (Wahl ExImpo AG, Gams, Switzerland) overnight. Excised gel slices were rehydrated in 100 µL 1× TE buffer (100 mM Tris, pH 7.5, 10 mM EDTA, pH 8.0) at 80°C for 10 min. Re-amplifications were carried out in 40 µL total volume with the following final reagent concentrations: 250 µM dNTPs, 1 µM BT7, 1 µM XSP6, 2.5 mM $MgCl_2$, 1.5 U/reaction *Taq* DNA polymerase and 5 µL eluate/reaction DNA. Cycling conditions were five cycles at 94°C for 45 s, 40°C for 60 s, 72°C for 60 s, followed by 35 cycles at high stringency (94°C for 60 s, 62°C for 60 s, 72°C for 60 s) and a 15-min extension step at 72°C. Sequencing was done using the dsDNA Cycle Sequencing System (Life Technologies). (A) DDRT-PCR using primers XTAT1 and XTAT2 in combination with DLP1–DLP4, DLPAT and DLPAG. cDNAs corresponding to circled bands have been re-amplified and sequenced. (B) Duplicate experiments (from duplicate RT) with XTAT1 (lanes 1–8), XTAT2 (lanes 9–16) and XAp11 (lanes 17–24) in combination with DLP1–DLP4. Identical signals occurring in Panel A are circled. (C) DDRT-PCR using primers XAp7–XAp12 in combination with DLP1–DLP4. "-": samples from untreated cells; "D": samples from dexamethasone-treated cells.

Two TAT-specific primers were used in combination with either four sets of DLP primers or with two separate DLPs (DLPAG and DLPAT). From whole display, bands corresponding to 14 up-regulated and 2 unchanged signals (Figure 2, A and B, circled, and Table 1) were re-amplified with primers XSP6 and BT7 and sequenced directly using primer XSP6. Out of the 14 differentially regulated signals, nine were found to correspond to TAT cDNAs with the approximative length expected from the given primer combinations (Figure 1B and Table 1). The deviation of a few nucleotides from the expected length and the number of signals generated from the same mRNA indicate that poly(A) addition does not happen at a precise distance from the polyadenylation signal. Three other bands were generated from metallothionein I mRNA and another fragment from α_2-macroglobulin, both of which are genes known to be regulated by glucocorticoids (6,8,12,14). One band corresponded to a novel gene whose regulated expression was confirmed by reverse Northern blot analysis (data not shown). The reproducibility was tested in a duplicate DDRT-PCR using the same combinations of primers (Figure 2B). The differential signals found in the first experiment were reconfirmed, the same for overall display patterns, although the intensity of corresponding signals in the two experiments was not always conserved. These results clearly demonstrate the validity of our modified technique. In particular, the identification of other differentially expressed genes, even with TAT-specific primers, shows that the increased primer length and presence of constant portions in the 5′ terminus do not impair or limit the priming ability. Primer specificity is determined by the last 3′ nucleotides in the primer. Moreover, the reliability of the method is greatly improved by a small percentage of false positives (13 out of 14 differential signals are shown to be true positives by sequence analysis).

DDRT-PCR was also performed with arbitrary primers XAp1–XAp20 in combination with DLP1–DLP4. Figure 2C displays examples of the patterns generated with XAp7–XAp12. XAp11 patterns are presented in duplicate (Figure 2B, lanes 17–24, and 2C) to emphasize again the reproducibility of the method. In the patterns partially shown in Figure 2C, 23 differential signals (12 up- and 11 down-regulated cDNAs) were found, and the identification of these fragments is currently in progress. Thus, differential display done with these arbitrary primers gave the usual patterns, including differentially expressed bands, excluding that our initial result with the primers XTAT1 and XTAT2 would be conditioned by the primer choice. This documents that our primer strategy does not impair, but actually improves, the initial strategy by Liang and Pardee. First, the use of universal re-amplification primers greatly facilitates and speeds up practical work. Second, the new primer design allows direct preparation of riboprobes for downstream analysis of re-amplified cDNAs and permits flexible cloning of fragments whenever required and direct sequencing with a unique primer.

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ANNEX II

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Cocaine-Induced Expression of the Tetraspanin CD81 and Its Relation to Hypothalamic Function

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CD81, a tetraspanin transmembrane protein involved in cell adhesion, was found by differential display to be up-regulated in the nucleus accumbens of rat brain following acute cocaine treatment (four injections of 30 mg/kg every 2 h followed by 24 h withdrawal). Cocaine-induced expression of CD81 in adult rat brain was confirmed by quantitative real-time RT-PCR. Its expression in neurons and its function in the brain are unknown. *In situ* hybridization shows a neuron-specific expression pattern in brain regions functionally related to the regulation of cardiovascular function and fluid homeostasis. CD81 displays codistribution to galanin and, to a lesser extent, to vasopressin. These findings add to data that suggest a connection between the brain reward pathway and the centers regulating endocrine and autonomic functions, in relation to neurochemical, behavioral, and somatic consequences of drug abuse.

INTRODUCTION

CD81 belongs to the tetraspanin family and contains four transmembrane domains with intracellular C- and N-terminal regions and one large extracellular loop. Tetraspanins form a multiprotein net on the cell surface that modulates integrin signaling and favors the formation of complexes of extracellular matrix proteins, ligands, and receptors, thus facilitating particular cellular responses to external stimuli (Maecker *et al.*, 1997; Yanez-Mo *et al.*, 1998; Berditchevski and Odintsova, 1999).

CD81, or TAPA-1[2], was first identified as the target of an antiproliferative antibody to a B-lymphoma cell line (Oren *et al.*, 1990). Subsequent studies showed that CD81 is involved in a large number of cellular functions, mostly established for cells of the immune system, including the regulation of cell shape, motility, or growth (Lin *et al.*, 1992; Boismenu *et al.*, 1996). CD81 is part of a complex on B lymphocytes, which includes CD19, CD21, and Leu13. This complex amplifies signal transduction through membrane-bound immunoglobulin, enabling B cells to respond to low concentrations of antigen by homotypic cellular aggregation. The CD81 component brings together independently functioning subunits, probably through integrin activation (Matsumoto *et al.*, 1993). Many tetraspanin multimolecular complexes contain a subset of integrins, but only CD81 and CD151 interact directly with integrins (Serru *et al.*, 1999). CD81 associates with integrins $\alpha3\beta1$, $\alpha4\beta1$, and $\alpha6\beta1$ (Mannion *et al.*, 1996), a functional relationship that may be cell type-specific (Behr and Schriever, 1995; Maecker *et al.*, 1997). CD81 also associates with MHC class II molecules in human B cells (Angelisova *et al.*, 1994), together with CD82, CD4, and CD8 (Imai and Yoshie, 1993). This provides a costimulatory signal with CD3 on human thymocytes (Todd *et al.*, 1996; Lagaudriere-Gesbert *et al.*, 1997). Recent studies found that

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[2] Abbreviations used: AD, anterodorsal thalamic nucleus; AVP, Arg-vasopressin; BST, bed nucleus of the stria terminalis; CART, cocaine- and amphetamine-regulated transcript; ChP, choroid plexus; CRF, corticotropin-releasing factor; DA, dopamine; DB, nucleus of the diagonal band of Broca; DIG, digoxigenin; Gal, galanin; Hipp, hippocampus; NAcc, nucleus accumbens; TO, oxytocin; PBS, phosphate-buffered saline; PVH, paraventricular hypothalamic nucleus; SN, substantia nigra; SO, supraoptic nucleus; SSC, saline sodium citrate; TAPA-1, target of the antiproliferative antibody-1; TH, tyrosine hydroxylase; VTA, ventral tegmental area.

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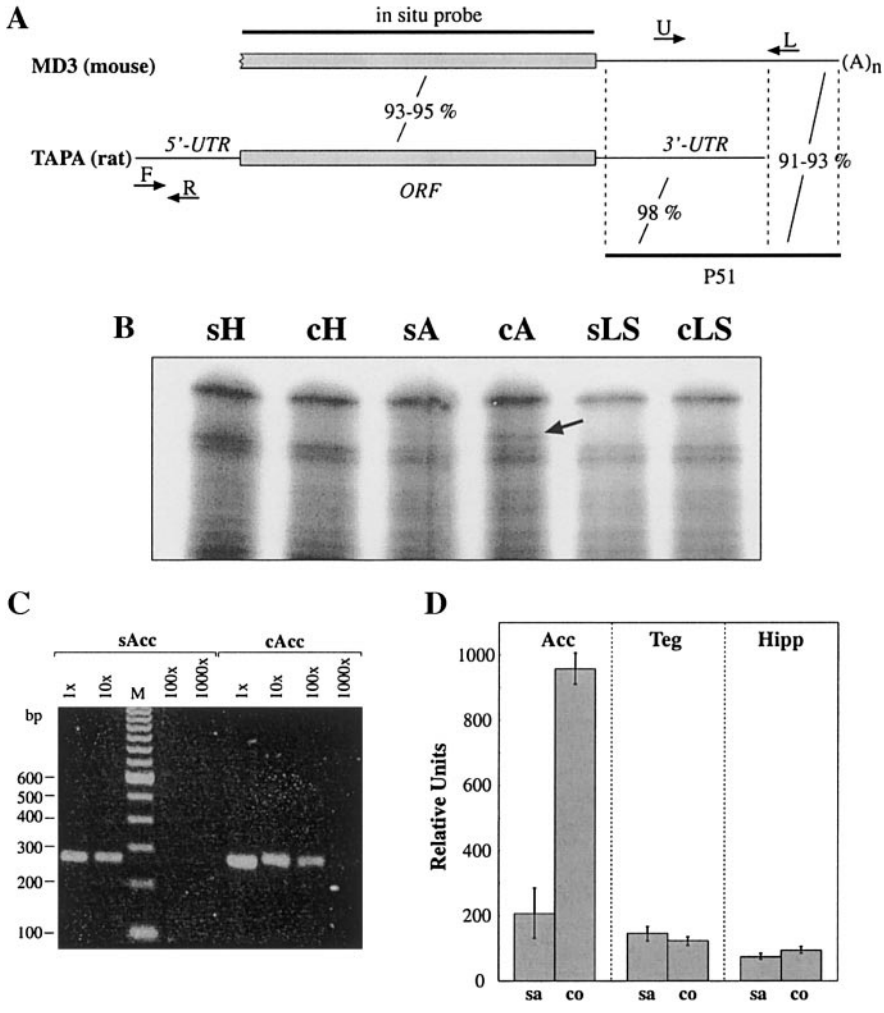

FIG. 1. (A) Map of CD81 and positions of fragments used in this study. MD3 and TAPA are the mouse and rat CD81 mRNA sequences, respectively, as designated in GenBank. Fragments are presented as submitted to GenBank, but both are incomplete, although full-length mRNAs from both species most probably have the same length and structure. Homology is shown between coding sequences of both mRNAs. The fragment P51 was found in the differential display study, with the larger part homologous to rat and mouse 3′-untranslated region (UTR) and the smaller one homologous only to mouse 3′-UTR, since the sequence for rat 3′-UTR is not yet available in GenBank. U and L show respectively the positions of the upper and lower primers used for end-point quantitative RT-PCR. F and R respectively indicate the positions of forward and reverse primers used for real-time PCR. The *in situ* probe was made from a clone containing the coding sequence of mouse CD81. ORF, open reading frame. (B) Differential display of mRNAs isolated from three brain regions of control or cocaine-treated rats (see text for details). s, saline; c, cocaine; H, hippocampus; A, nucleus accumbens; LS, lateral striatum. The band corresponding to CD81 is indicated by an arrow. (C) End-point quantitative RT-PCR with CD81-specific primers on cDNA produced from mRNA of saline- or cocaine-treated rat accumbal tissue (sAcc and cAcc, respectively). Four serial dilutions of cDNA were used (from 1× to 1000×), and the PCR products were loaded on gel together with 100 bp ladder. The CD81 amplified band of 243 bp disappears when PCR is performed on 100× sAcc dilution, while is still present in cAcc, indicating upregulation of CD81 upon cocaine treatment in NAcc. (D) Real-time quantitative RT-PCR with CD81-specific primers on cDNA produced from NAcc, tegmentum, or hippocampus. Total RNA was extracted, using an RNAqueous kit (Ambion), from the NAcc of saline- or cocaine-treated rats. Two independent RT reactions were performed and the cDNA templates used for PCR. Real-time quantitative PCR was carried out on an iCycler (Bio-Rad) using the SYBR green method, with appropriate primers for CD81 and for 28S rRNA (as a control), as described under Experimental Methods. Up to five independent PCRs were performed for each condition. The threshold cycle was defined as the fractional cycle number at which the fluorescence passed the fixed threshold. The measures of relative fluorescence were made during the threshold cycle, determined for each PCR. The measures for CD81 were normalized using the numbers for 28S rRNA. The average with standard deviation is presented. Although the comparison of absolute values between different regions is not possible, these results clearly demonstrate the CD81 upregulation in the cocaine-treated NAcc, as opposed to tegmentum and hippocampus.

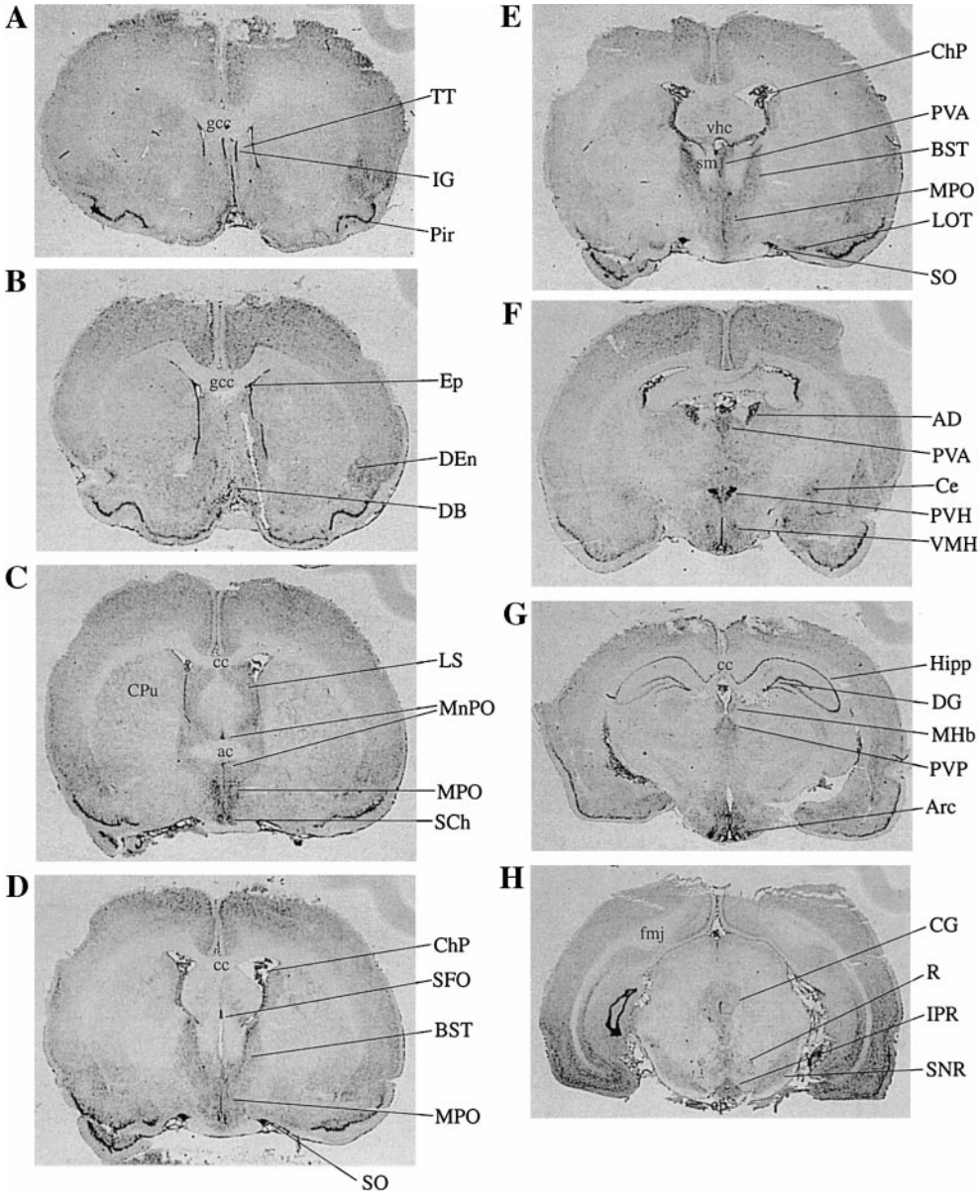

FIG. 2. *In situ* hybridization of CD81 mRNA distribution throughout the adult rat brain. Photomicrographs are shown for coronal 25-μm sections hybridized with DIG-labeled CD81 probe. (A–H) Sections are shown in rostral-to-caudal direction. Regions specifically labeled with CD81 probe are marked on the right side. For orientation, some unstained regions are indicated on sections. AD, anterodorsal thalamic nucleus; Arc, arcuate nucleus; BST, bed nucleus of the stria terminalis; CG, central gray; ChP, choroid plexus; DB, nucleus of the diagonal band of Broca; DEn, dorsal endopiriform nucleus; DG, dentate gyrus; Ep, ependymal layer of the ventricles; IG, induseum griseum; IPR, interpeduncular nucleus; Hipp, hippocampal formation; LOT, nucleus of the lateral olfactory tract; LS, lateral septum; MHb, medial habenular nucleus; MnPO, median preoptic hypothalamic nucleus; MPO, medial preoptic hypothalamic nucleus; Pir, piriform cortex; PVA, paraventricular thalamic nucleus, anterior part; PVH, paraventricular hypothalamic nucleus; PVP, paraventricular thalamic nucleus, posterior part; R, red nucleus; SCh, suprachiasmatic nucleus; SFO, subfornical organ; SN, substantia nigra; SO, supraoptic nucleus; TT, tenia tecta; VMH, ventromedial hypothalamic nucleus; ac, anterior commissure; cc, corpus callosum; CPu, caudate putamen; fmj, forceps major of the corpus callosum; gcc, genu of the corpus callosum; sm, stria medullaris of the thalamus; vhc, ventral hippocampal commissure.

human CD81 binds to hepatitis C virus via the envelope protein E2 and is, therefore, a possible key molecule in virus pathogenicity (Pileri *et al.*, 1998). Anti-CD81 blocks T cell maturation (Boismenu *et al.*, 1996); however, in CD81-deficient mice the thymocytes develop normally (Tsitsikov *et al.*, 1997). These mice show, in

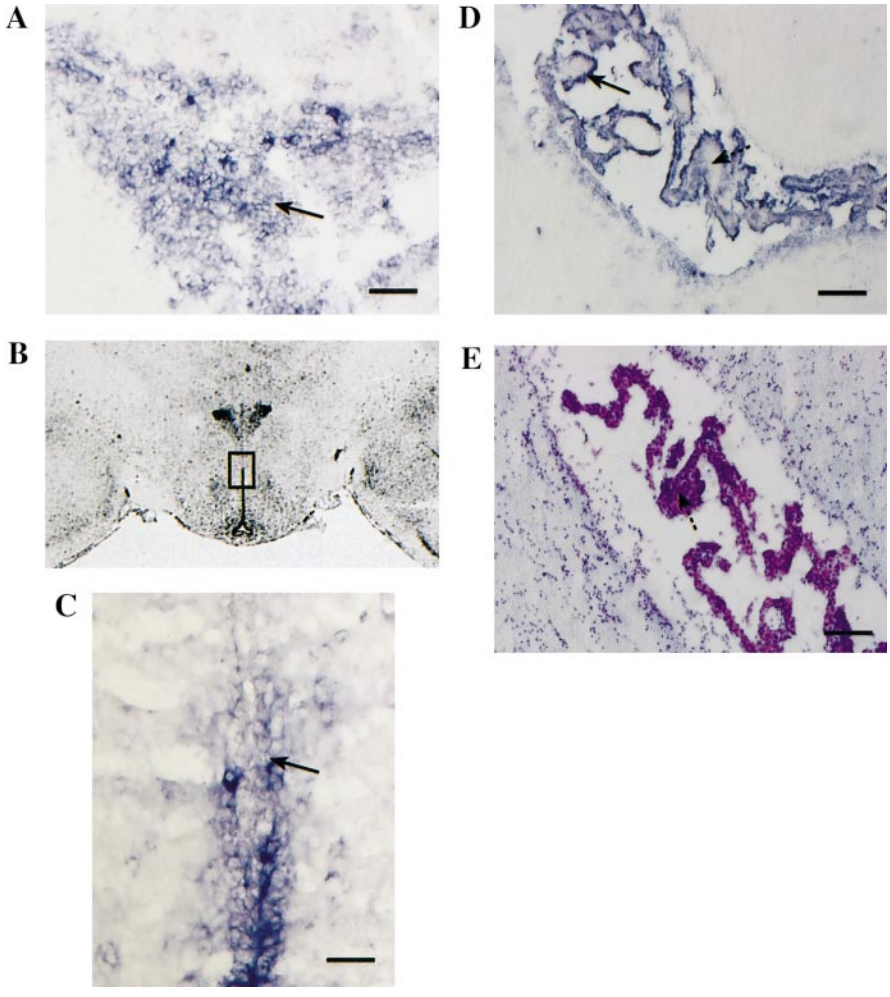

FIG. 3. CD81 *in situ* hybridization in the ependymal layer of the ventricles and choroid plexus. (A) The ependymal lining of the lateral ventricle. Several layers of CD81-positive ependymal cells are indicated by an arrow. (B) The ependymal lining of the third ventricle at the level of anterior hypothalamus. Inset magnified in (C): The ependymal lining of the third ventricle. The border between CD81-positive chain-like ependymal cells and CD81-negative zones is indicated by arrow. (D) CD81 signal in choroid plexus. (E) Nissl staining in choroid plexus of section adjacent to D. Cells facing the ventricular cavity are indicated by arrows, internal choroid plexus cells by dotted arrows. Scale bars, 50 μm (in A, D, E) and 25 μm (in C).

general, a subtle phenotype, suggesting the extensive compensatory processes that occur during development (Levy *et al.*, 1998).

CD81 is expressed in virtually all types of cells, with the exception of red blood cells and platelets (Levy *et al.*, 1998), and has been thoroughly characterized in lymphocytes. In the brain CD81 is expressed by glial cells (Sullivan and Geisert, 1998), anti-CD81 causing changes in astrocyte morphology (Geisert *et al.*, 1996). CD81 is upregulated during early postnatal development, at the time of glial birth and maturation, and in the case of reactive gliosis (Irwin and Geisert, 1993; Sullivan and Geisert, 1998). This upregulation is evidence for a role of tetraspanins in brain plasticity.

In this study we have performed a differential display screening to identify molecular cues induced by psychomotor stimulants that revealed CD81 upregulation in the nucleus accumbens (NAcc) upon cocaine treatment. We have confirmed this finding by quantitative RT-PCR and undertaken a detailed description of CD81 distribution in rat brain. This shows that CD81 is not exclusively glial and is expressed in selective brain nuclei, mainly confined to regions important for regulation of cardiovascular function and fluid homeostasis. The pattern of CD81 localization is consistent with a putative role in relation to effects observed after drug withdrawal. This adds to data linking together the neurochemical, behavioral, and somatic consequences of

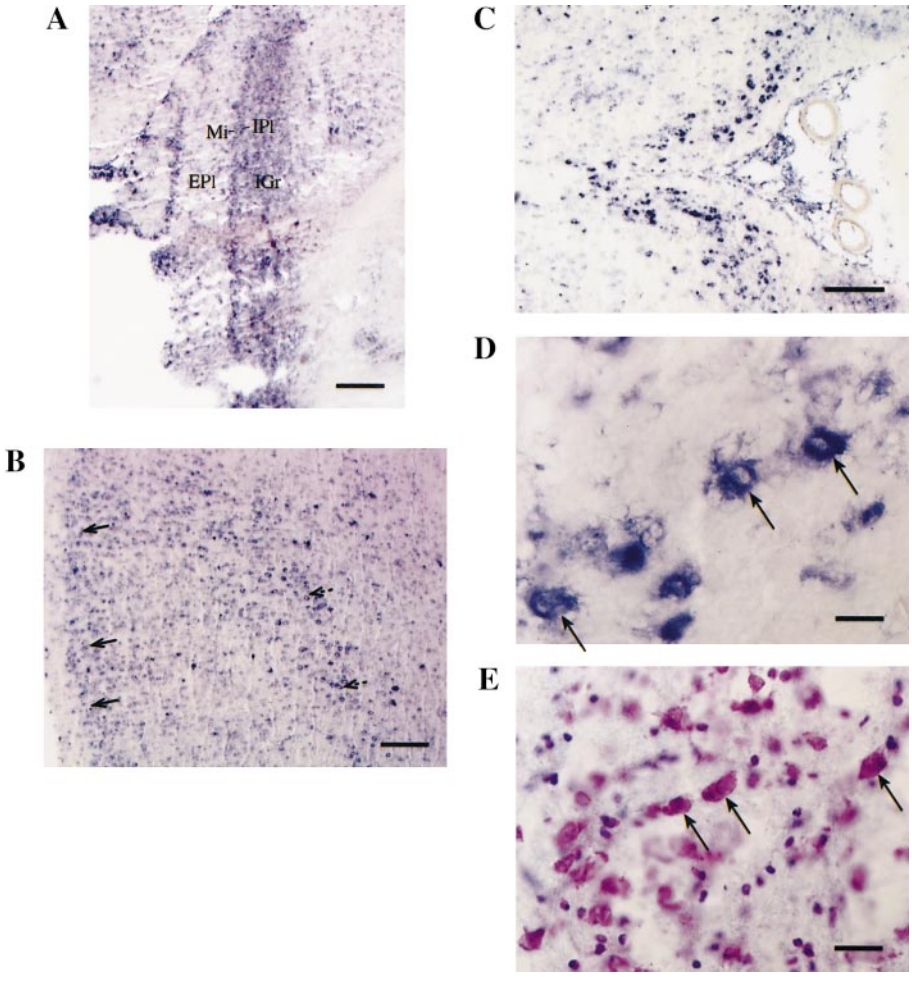

FIG. 4. CD81 *in situ* hybridization in the olfactory bulb (A) and the cortex (B) and the diagonal band of Broca. EPl, external plexiform layer; IGr, internal granular layer; IPl, internal plexiform layer; Mi, mitral cell layer. The small densely packed cells of layer II are indicated by arrows; the bigger diffusely distributed cells of layers IV–V are marked with dotted arrows. (C–E) CD81 *in situ* hybridization in the diagonal band of Broca. (C) Low magnification; (D) high magnification. (E) A Nissl-stained adjacent section. Big cells positive for CD81 are indicated by arrows in both stainings. Scale bars, 250 μm (in A–C) and 25 μm (in D, E).

drug abuse. It provides additional evidence for a relation between the primary targets of psychostimulants in the brain reward circuits and centers regulating autonomic or neuroendocrine functions.

RESULTS

CD81 Upregulation in Nucleus Accumbens

To examine the changes in gene expression in the dopaminergic brain system upon acute cocaine treatment, we used the method of differential display (Liang and Pardee, 1992) according to the modifications described recently (Brenz Verca *et al.*, 1998). Patterns of expression were compared for mRNAs isolated from the NAcc, the lateral striatum, and the hippocampus (Hipp) (as control) of saline- or cocaine-treated rats.

Among differentially expressed tags one cDNA was found to be induced upon cocaine treatment in the NAcc (Fig. 1B). The candidate was not detectable in other parts, neither in the NAcc of control animals nor in any other brain region examined. The result was reproduced in triplicate differential display experiments starting from three independent reverse transcription (RT) reactions. The band was cut out and reamplified with the set of universal reamplification

primers described in the protocol (Brenz Verca *et al.*, 1998) and the PCR product was sequenced directly, yielding a sequence with 98% homology with the TAPA-1 (Geisert *et al.*, 1996), rat CD81-homologous sequence.

To further assess the induction of CD81, primers were designed for quantitative PCR based on rat (TAPA-1) and mouse (MD3) CD81 homologous sequences in GenBank (Fig. 1A). Serial 10-fold dilutions of RT product from mRNA of saline- or cocaine-treated rats were amplified with these CD81-specific primers. As a control, the product of the RT reaction was used but without RTase added. The expected band of 243 bp was found in undiluted or 10-fold-diluted cDNAs from both treated and untreated animals; however, the signal from 100-fold-diluted cDNA was evident only for cocaine-treated brain, suggesting an increased quantity of CD81 mRNA in this region compared to control (Fig. 1C). At the 1000-fold dilution, the band was absent under both conditions.

The quantification of RT-PCR end-product does not allow for precise evaluation of concentration differences between the samples. Therefore the real-time RT-PCR was used to confirm the upregulation of CD81 mRNA in the NAcc, which allows for quantification of PCR product during the exponential phase of reaction. Primers were chosen based on the rat (TAPA-1) CD81-homologous sequence in GenBank (Fig. 1A). Quantitative PCR was performed in quintuplicate from two different RT reactions and 28S rRNA was used as a normalization standard in all experiments. As shown in Fig. 1D, CD81 is upregulated after cocaine treatment and its expression raises 4.6-fold in the NAcc. No changes in CD81 expression were found in the Hipp and the tegmentum. Controls in which RTase had been omitted or in which just one primer had been used in the PCR were always negative. These data clearly indicate that CD81 upregulation is specific for certain brain regions and that CD81 may play a major role in the response to cocaine.

Localization of CD81 mRNA in the Rat Brain

We performed *in situ* hybridization with a riboprobe corresponding to the coding sequence of CD81 mRNA (Fig. 1A). Controls with sense riboprobe consistently yielded no signals. CD81 distribution throughout the brain is shown in Fig. 2 on coronal sections in a rostral-to-caudal direction.

In general CD81 expression is abundant in typically glial structures, confirming the immunocytochemical observations made by Geisert and colleagues (Sullivan and Geisert, 1998). The *glia limitans*, the layer of astrocytic cells lying beneath the pial surface, was positive for CD81 (not shown). However, only the minor part of these cells was stained, as could be seen by comparison with Nissl-stained consequent sections (not shown). The same was true for the ependymal lining of ventricles. In many cases one or more layers of ependymal cells were stained at the inner surface of the ventricles (Figs. 3A and 3B). However, we have found some exceptions to this general rule. As can be clearly seen in Figs. 3B and 3C, the extensive staining is present in the ventral part of the third ventricle where the tightly joined, labeled ependymal cells form a chain-like structure, though in the more dorsal part of the ventricle, the staining disappears completely. We also found the CD81 signal in circumventrical organs, namely in the choroid plexus (ChP) (Fig. 2E) and the subfornical organ (Fig. 2D). While comparing the two subsequent sections, stained for CD81 mRNA and Nissl substance, respectively (Figs. 3D and 3E), we observed that the staining for CD81 in ChP was especially strong for the external layer of cells facing the ventricular cavity filled with cerebrospinal fluid. However, the internal cells of the ChP were unstained for CD81.

In addition to these purely glial structures, we could find other positively stained regions, not described thus far, where the staining was not equivocally confined to glial or neuronal cells.

Olfactory areas. In the olfactory bulb, the internal granular layer was most extensively labeled with CD81, whereas both the external and the internal plexiform layers as well as the mitral cell layer were moderately stained (Fig. 4A). Of all other regions related to the olfactory system, the piriform cortex and the induseum griseum were the most intensively labeled (see, for example, Fig. 2A). Less CD81 staining was found in the anterior olfactory nucleus (not shown), tenia tecta (Fig. 2A), dorsal endopiriform nucleus (Fig. 2B), and nucleus of the lateral olfactory tract (Fig. 2E), and a very faint signal was seen in the olfactory tubercle.

Cortex. The parietal cortex was the least intensively marked zone, compared to the other cortical regions (see, for example, Fig. 2B). At the cellular level, CD81 expression was absent in cortical layer I, most pronounced in layer II with small, quite densely packaged cells, and then equally distributed in lower cortical layers, where the cells were in general larger and more diffusely distributed (Fig. 4B).

Septum. The posterior septum was not labeled with CD81 probe. The lateral septum was moderately labeled throughout, including its dorsal, intermediate,

and ventral parts (Fig. 2C). However, the most specific CD81 labeling was found in the medial septal division. The nucleus of the diagonal band of Broca (DB) was extensively labeled (Figs. 2B and 5C). Only a very specific restricted subset of cells was positive for CD81, as seen by comparison with a Nissl-stained subsequent section (Figs. 4D and 4E). Nissl staining clearly shows at least two different cell types, small and large ones (Fig. 4E), and CD81 staining is almost exclusively confined to large cells, probably cholinergic and/or peptidergic neurons. Small cells are either GABAergic interneurons or astrocytes and are CD81-negative, so it is very improbable that expression is confined to glial cells in that region.

Hippocampus. Moderate CD81 staining was found in the pyramidal cell layer of the hippocampal formation, as well as in the granule cell layer of dentate gyrus (Fig. 2G).

Amygdala and extended amygdala. Moderate CD81 expression was found in the bed nucleus of the stria terminalis (BST) (Fig. 2D) and in the central amygdaloid nucleus (Fig. 2F). Other amygdala subdivisions contained very few CD81 message-positive cells.

Thalamus. Faint CD81 staining of mainly medium-sized neurons was found in medial habenular (Fig. 2G), paraventricular (Figs. 2E–2G), and reuniens (not shown) thalamic nuclei. However, more specific and extensive labeling was in the anterodorsal nucleus (AD) (Figs. 2F and 6A). These neurons were bigger and rounded and contained large nuclei (Fig. 5C). Interestingly, the AD contains the highest density of large-size neurons compared to other thalamic subregions. As a result of this morphological particularity the borders of the nucleus can be easily appreciated in Nissl-stained sections even at low magnification (Fig. 5B). Some cells show a specific triangular pattern of CD81 labeling, possibly reflecting mRNA concentration in a region of the axon hillock (Fig. 5C).

Hypothalamus. In the preoptic regions, the median preoptic nucleus was highly positive for CD81 mRNA labeling (Fig. 2C). Both the medial preoptic area and the medial preoptic nucleus were moderately stained (Figs. 2C–2E). In the periventricular zone, the suprachiasmatic nucleus was moderately labeled (Fig. 2C), and arcuate (Fig. 2G) and paraventricular (PVH) (Figs. 2F and 7B) nuclei were both very extensively stained. The specificity was pronounced in PVH with much higher labeling in the lateral magnocellular part compared to medial parvocellular and ventral parts (Fig. 6B). The massive CD81 staining in the lateral part is probably confined to magnocellular neurosecretory cells (Fig. 6D). Again some cells show the triangular pattern of CD81 labeling in the area of the axon hillock. In the supraoptic nucleus (SO), expression is also probably confined to magnocellular neurosecretory cells (Figs. 7A and 7B). In the tuberal region, the ventromedial nucleus is slightly labeled (Fig. 2F). In the mammillary region, the supramammillary and lateral mammillary nuclei are slightly labeled (not shown). In general, disperse staining was present throughout the entire hypothalamus.

Brain stem. Several regions were moderately labeled in brain stem, including the central gray area, the interpeduncular nucleus, the substantia nigra (SN), and the linear raphe nucleus (Fig. 2H).

Colocalization of CD81 and Tyrosine Hydroxylase Messages in Rat Brain as Shown by in Situ Hybridization

The modified expression of CD81 message following cocaine administration and in regions related to dopaminergic brain system suggested that, under normal conditions, CD81 might be localized in dopaminergic neurons. Therefore we compared the CD81 distribution with one for tyrosine hydroxylase (TH) in subsequent brain sections. Both CD81 and TH staining were often found in the same brain regions, including olfactory bulb, hypothalamic nuclei, and SN. However, TH is more specific and touches only very limited subgroups of cells, for example in SO (Figs. 7B and 7C) and SN (data not shown). Without double-labeling experiments, it cannot be said as a general rule that both messages are colocalized, especially with our observations that CD81 is not at all expressed in the A10 and A11 groups of dopaminergic cells, nor is TH expressed in many CD81-positive areas.

CART Overexpression in the Nucleus Accumbens of Cocaine-Treated Rat Brain

The CART (cocaine and amphetamine-regulated transcript) peptide is a putative neurotransmitter and trophic factor. CART is one of the messages known to be overregulated in the striatum upon acute psychostimulant application. More precise localization and functional significance of such overexpression have yet to be demonstrated. However, such a signal could be still present 24 h after acute cocaine treatment, when the expression of acutely induced, immediate early genes is already terminated. Thus, we decided to use the CART probe as a positive control of cocaine-induced gene expression in our *in situ* hybridization studies. As expected, CART message was overexpressed in the stria-

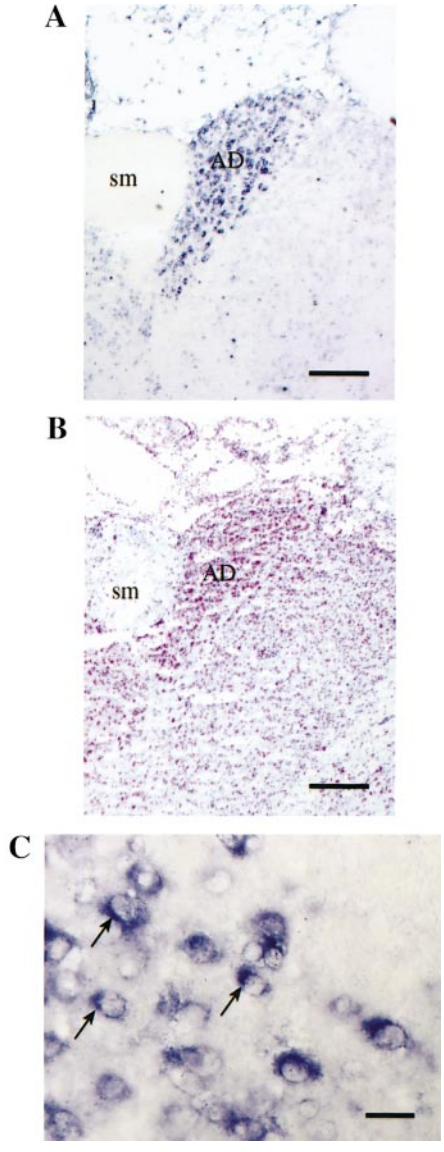

FIG. 5. CD81 *in situ* hybridization in the anterodorsal nucleus of the thalamus. (A) Low magnification; (B) an adjacent Nissl-stained section; (C) CD81 staining at high magnification. Staining of axon hillocks is indicated by arrows. AD, anterodorsal thalamic nucleus; sm, stria medullaris of the thalamus. Scale bars, 250 μm (in A, B) and 25 μm (in C).

tum 24 h after acute cocaine (data not shown). Moreover, we have shown that this overexpression is specifically confined to the ventral part of striatum, or the NAcc. The signal seemed to be particularly strong in the shell of the NAcc; however, this statement needs additional experimental support. Concerning the general expression of CART message in rat brain, we observed localization similar to that found in previously published results (Douglass *et al.*, 1995), namely in olfactory areas, PVH (Fig. 6C), and SO, but we could not find any signal in the central amygdala region (Fagergren and Hurd, 1999).

CD81 and CART Colocalization in Brain

Because both CD81 and CART were found to be overexpressed upon cocaine treatment in the NAcc, it was interesting to compare their general distribution patterns in adult rat brain. In fact, we could observe both signals in different olfactory regions, including tenia tecta, induseum griseum, anterior olfactory nucleus, and piriform cortex. In addition, we found both messages in particular hypothalamic regions, including the PVH, arcuate, and SO nuclei, which are highly heterogeneous at the cellular level, even though they have a common function in the regulation of endocrine and autonomic functions. However, whereas CD81 localization was primarily confined to the magnocellular part of the PVH (Fig. 6B), CART signal was preferentially concentrated in the parvocellular region (Fig. 6C). Another striking difference in hybridization signal distribution of the two probes was in the basal ganglia region. Whereas CART mRNA is found there in very high concentration, especially in the NAcc, ventral pallidum, and globus pallidus, CD81 was not expressed in any of these regions.

DISCUSSION

CD81 Is Involved in Cocaine Action

In the present study we have shown the upregulation of CD81 in the NAcc following cocaine treatment. The expression was region-specific and not observed in other regions examined (lateral striatum, tegmentum, and Hipp). The absence of CD81 induction in the tegmentum suggests that the effect is mediated by cocaine action on postsynaptic part of the ventral tegmental area (VTA)–NAcc axis, probably involving dopamine (DA) receptors in the NAcc. The DA hypothesis of action is fortified by the fact that DA and its metabolite levels are increased specifically in the NAcc of CD81-deficient mice, as opposed to other neurotransmitters (Michna *et al.*, 1999; submitted for publication).

Although we found CD81 in classical glial structures (ChP, ependyma, glia limitans) in accordance with previously published data (Sullivan and Geisert, 1998), it is not exclusively glial, as many other groups of CD81-

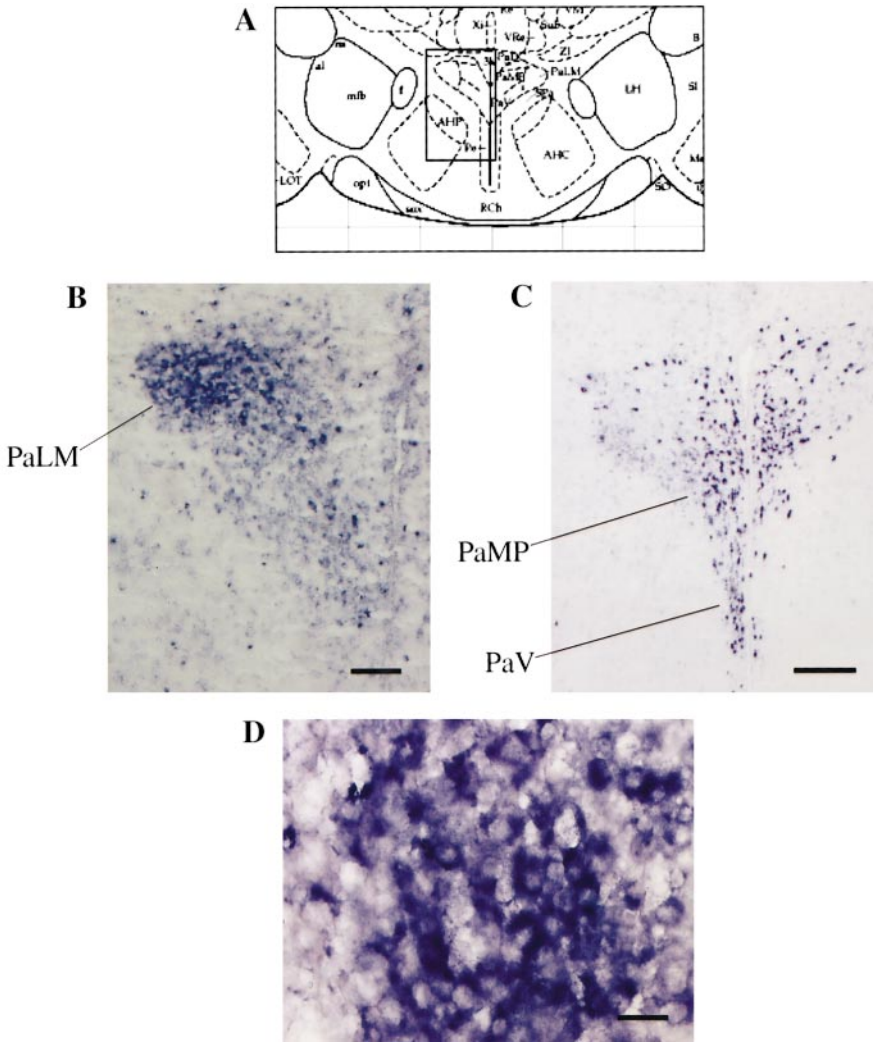

FIG. 6. CD81 *in situ* hybridization in the paraventricular hypothalamic nucleus. (A) Schematic view of coronal rat brain section in the region of anterior hypothalamus, with inset represented in CD81 (B) and CART (C) hybridized micrographs. (D) CD81-positive cells in PaLM at high magnification. PaLM, lateral magnocellular part; PaMP, medial parvocellular part; PaV, ventral part. Scale bars, 100 μm (in B), 250 μm (in C), and 25 μm (in D).

positive cells in the PVH, SO, or DB show a typically neuronal morphology.

CD81 Is Associated with Circuits Controlling Cardiovascular Responses and Fluid Homeostasis

Our study shows high expression of CD81 in brain circuits involved in cardiovascular responses and fluid homeostasis. These circuits consist of two parts: (a) the sensory part composed of ChP, subfornical organ, vascular organ of lamina terminalis, and median preoptic nucleus and containing receptors for insulin, vasopressin (AVP), atrial natriuretic factor, and angiotensin II and (b) the motor or effector part composed of the magnocellular cells of PVH and SO in hypothalamus secreting AVP and oxytocin (TO), two hormones influencing body fluid homeostasis through regulation of blood pressure as well as through water retention by the kidney (Tohyama and Takatsuji, 1998). In these hypothalamic nuclei, but also in other AVP-positive areas (the suprachiasmatic nucleus, DB, BST, and Hipp) or in regions projecting to PVH and SO (DB, BST, and medial preoptic nucleus), CD81 is expressed at higher levels. Together these results suggest a functional role

for CD81 in the neuroendocrine regulation of cardiovascular and fluid homeostasis.

CD81 Is Associated with Galanin Pathways

We found striking similarities in brain localization of CD81 and galanin (Gal) (Table 1). CD81 and Gal colocalization specificity is maintained within the same region: in PVH, for example, both are strongly expressed in the lateral part and weakly in the medial and ventral parts (see Table 1). The specificity was striking in the thalamus where high expression of both Gal and CD81 is found in only one thalamic nucleus, AD of anterior group, a part of limbic Papez's circuit (Heimer, 1995). AD inhibits the hypophysoadrenal system under basal and acute stress conditions (Suarez *et al.*, 1998), again providing a link of CD81 to neuroendocrine function.

From our study it is difficult to say if CD81 and Gal are colocalized at the cellular level, without double-labeling experiments. However, the expression of CD81 in a subset of DB and AD neurons with the characteristic size of peptidergic neurons strongly favors this hypothesis.

Gal modulates the dopaminergic function. Direct interaction between Gal and the DA pathway leads to rapid increase of prolactin and growth hormone secretion following intraventricular injections of Gal (Melander *et al.*, 1987). Gal release from noradrenergic neurons of locus coeruleus (Hokfelt *et al.*, 1999) inhibits the activity of VTA dopaminergic cell bodies, thus indirectly interfering with the reward pathway. This results in two of the principal symptoms seen in depression, decreased motor activation and anhedonia (Weiss *et al.*, 1998). The codistribution of CD81 and Gal indicates functional relations between the two molecules and could explain CD81 involvement in drug addiction. CD81, for example, may provide a link between the first site of cocaine action (NAcc) and the Gal pathway, ultimately altering dopaminergic responses and influencing the reward pathway. The involvement of CD81 in long-lasting cocaine effect is supported by the observation of modified cocaine-elicited behavior in CD81-deficient mice (Michna *et al.*, 1999; submitted for publication).

Cocaine Interacts with Neuroendocrine Function

The cocaine-induced expression of CD81 and its putative role in neuroendocrine function provide additional evidence of an interaction between hormonal function and the actions of drugs of abuse (Torres and Rivier, 1992). The best examples of such interaction include reciprocal cocaine regulation of the HPA axis and OT–AVP system.

Cocaine induces changes in the HPA axis, often in "binge" protocols very similar to the one used in the present study. Acute binge cocaine alters hypothalamic and extrahypothalamic/limbic corticotropin-releasing factor (CRF) concentrations in rats (Sarnyai, 1998), leading to the release of adrenocorticotropic hormone, β-endorphin, and corticosterone and activation of the HPA axis (Moldow and Fischman, 1987; Zhou *et al.*, 1999). Chronic binge cocaine administration is coupled to CRF decrease in the hypothalamus and activation of CRF gene expression in extrahypothalamic regions, which has implications in the behavioral responses to cocaine (Zhou *et al.*, 1996). Finally, cocaine withdrawal elicits

TABLE 1
The Codistribution of CD81 and Galanin in Rat Brain

Brain region	CD81	Galanin
Olfactory system		
Olfactory bulb	+	+/−
Anterior olfactory nucleus	+	+
Cortex	+	+
Septum		
Nucleus of the diagonal band +++	+++	++
Hippocampus	++	+
Thalamus		
Paraventricular nucleus	+	+
Anterodorsal nucleus	+++	++
Amygdala and extended amygdala		
Bed nucleus of the stria terminalis	++	++
Hypothalamus		
Suprachiasmatic nucleus	+	+
Arcuate nucleus	++	++
Supraoptic nucleus	+++	+++
Periventricular nucleus	+	++
Paraventricular nucleus, lateral magnocellular part	+++	+++
Paraventricular nucleus, medial parvocellular part	+	+
Paraventricular nucleus, ventral part	+	+
Medial preoptic area	+/−	+
Medial preoptic nucleus	++	++
Anterior hypothalamic area +	+	+
Lateroanterior nucleus	+	+
Tuber cinereum area +	+	++
Accessory neurosecretory nuclei	+++	+++
Brain stem		
Interpeduncular nucleus	+	+

Note. CD81 expression was assessed in the present study. We have arbitrarily defined four grades of *in situ* hybridization signal in our reactions (from "+/−" to "+++", according to its intensity under light microscopy at high magnification). The data on galanin localization were taken from the published sources (Hamill *et al.*, 1986; Melander *et al.*, 1986).

anxiety-like behavior and alterations of CRF concentration in the hypothalamus, amygdala, and basal forebrain (Sarnyai, 1998).

Exposure to stress increases vulnerability to self-administer psychostimulants. Plasma corticosterone is necessary for cocaine reinforcement. The mechanism by which corticosteroids interact with the mesocorticolimbic dopaminergic system remains to be determined, but the medial prefrontal cortex is probably one brain region where DA and adrenocorticosteroids interact to affect cocaine reinforcement (Goeders, 1997).

Both acute and chronic cocaine administration induces changes in the OT and AVP contents in the hypothalamus and in limbic structures (Van de Kar *et al.,* 1992). AVP inhibits, whereas OT facilitates, the development of behavioral sensitization to cocaine (Sarnyai, 1998). OT also inhibits acute cocaine-induced locomotor hyperactivity, exploratory activity, and stereotyped behavior in rodents. Interaction of OT with dopaminergic neurotransmission in the NAcc has been postulated as a mechanism of action to modulate neuroadaptation to cocaine (Sarnyai, 1998).

A more recent example concerns the CART upregulation in the striatum after acute cocaine treatment (Douglass *et al.,* 1995). CART is involved in the regulation of feeding behavior, sensory processing, and stress (Couceyro *et al.,* 1997, 1998; Kuhar and Dall Vechia, 1999). CART expression was also observed in our protocol of cocaine treatment. We have found the expression of CART and CD81 mRNAs in functionally separate hypothalamic cell groups, suggesting a distinct role for the two proteins in normal and cocaine-treated brain.

These studies strongly support the hypothesis that cocaine action affects expression of proteins important for hypothalamic functions, such as cardiovascular and fluid homeostasis control, stress response, and feeding behavior.

In the present study CD81 was found to be upregulated in the NAcc upon cocaine treatment. DA level is increased in the NAcc of CD81-deficient mice, and their behavioral responses to cocaine are modified (Michna *et al.,* 1999; submitted for publication). In addition we show the expression of CD81 in brain regions functionally related to the regulation of cardiovascular function and fluid homeostasis and its codistribution with Gal and AVP. These findings add to previous data suggesting a connection between the brain reward pathways and the centers regulating endocrine and autonomic functions and may show a relation of the neurochemical, behavioral, and somatic consequences of drug abuse.

EXPERIMENTAL METHODS

Drug treatment. Eight-week-old, male Sprague–Dawley rats (Taconic Farms, Germantown, NY) were injected subcutaneously with saline or 30 mg/kg cocaine–HCl (Sigma Chemical Co., St. Louis) every 2 h for four injections and were sacrificed 24 h after the last injection (Gawin, 1991). Brains were extracted and striatum, hippocampus, and tegmentum were dissected as follows: a coronal slice through the caudal third of the olfactory tubercle was made. A second coronal slice was made through the anterior hypothalamus. Lateral striatum and accumbens were isolated from the resulting coronal section. Accumbens tissue around the anterior commissure was first dissected, followed by striatal tissue to the corpus callosum. On exposure of the dorsal side of the remaining undissected mid- and hindbrain, the telencephalon was pushed forward and the Hipp lifted free. At the leading edge of the superior colliculus, a diagonal coronal incision was made in the rostral-to-caudal direction. Similarly a caudal-to-rostral cut was made at the trailing edge of the inferior colliculus. The resulting wedge of tissue contains the colliculi on its dorsal face and a section of the cerebral peduncle on its ventral face. Recumbent on its posterior plane, the peduncle was removed and a vertical incision was made ventral to the cerebral aqueduct. The resulting strip of tegmentum contained the cell bodies of the midbrain dopaminergic system.

RNA isolation and differential display analysis. Total RNA was extracted, with the RNAqueous kit (Ambion), from the selected brain regions of control or cocaine-treated rats. Differential display was then performed essentially as described (Brenz Verca *et al.,* 1998). Briefly, 1 μg total RNA was reverse transcribed with the modified oligo(dT) primer sets. Reaction conditions were set as described (Liang and Pardee, 1992). Subsequent PCR was performed with the same sets of oligo(dT) primers in addition to arbitrary primers XAp1 to XAp20. PCR was performed as follows: 5 cycles at low stringency (94°C for 60 s, 40°C for 120 s, 72°C for 60 s) followed by 35 cycles at high stringency (94°C for 45 s, 56°C for 60 s, 72°C for 60 s). A typical PCR reaction was carried out in 20 μl final volume with the following final reagent concentrations: dNTPs, 2 μM; 3′-anchoring primer, 1 μM; arbitrary primer, 1 μM; $MgCl_2$, 1 mM. One-fiftieth of the cDNA obtained from RT, 1–2 μCi [α-^{32}P]dATP at 3000 Ci/mmol (Hartmann Analytic), and 1.5 U *Taq* polymerase (Gibco BRL) were used for each PCR. Reaction products were resolved on 6% denaturing sequencing gels which were dried and ex-

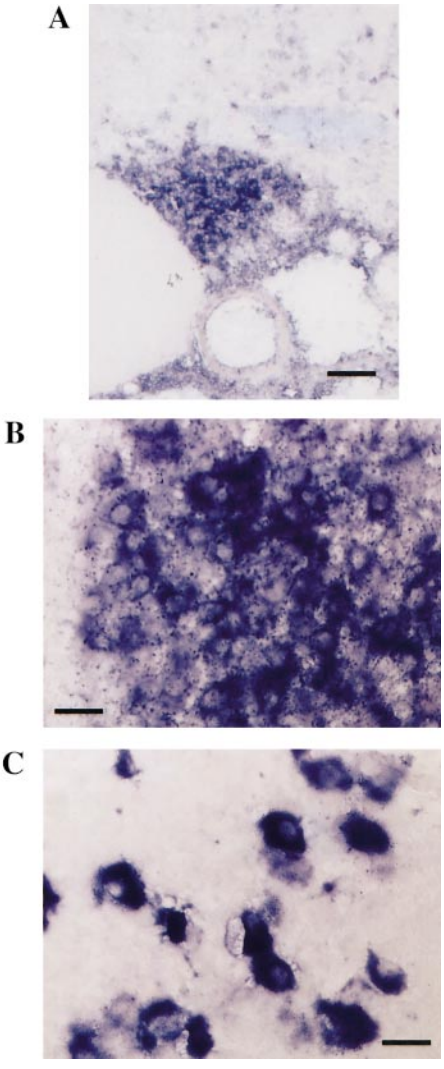

FIG. 7. CD81 *in situ* hybridization in the supraoptic magnocellular cells at (A) low magnification and (B) high magnification. (C) TH hybridization at the same magnification as in (B). Scale bars, 100 μm (in A) and 25 μm (in B, C).

posed to Fuji X-ray film overnight. Excised gel slices were rehydrated in 100 μl 1× TE at 80°C for 10 min. Reamplifications were carried out in 40 μl total volume with the following final reagent concentrations: dNTPs, 250 μM; reamplification primers, 1 μM each; $MgCl_2$, 2.5 mM; *Taq* DNA polymerase, 1.5 U / reaction; DNA, 5 μl eluate / reaction. Cycling conditions were 5 cycles (94°C for 45 s, 40°C for 60 s, 72°C for 60 s), followed by 35 cycles at high stringency (94°C for 60 s, 62°C for 60 s, 72°C for 60 s) and a 15-min extension step at 72°C. PCR products were sequenced by Microsynth GmbH either directly or after cloning into pBluescript KS(+) vector.

Quantitative end-point RT-PCR. Total RNA was extracted using an RNAqueous kit (Ambion, AMS Biotechnology, Ltd., Lugano, Switzerland) from the NAcc of control or cocaine-treated rats. Reverse transcription was performed essentially as described (Liang and Pardee, 1992; Brenz Verca *et al.*, 1998). Primers for PCR were designed based on CD81 mRNA GenBank sequence for rat (TAPA-1) and mouse (MD3). The upstream primer was 5′-ACT TTT CCT GTC ACC TTT TGG-3′; the downstream primer was 5′-TCT TGA AAT GGA GGC AGG AAA-3′. PCR was carried out in 20 μl final volume with the following final reagent concentrations: dNTPs, 250 μM; both primers, 1 μM; $MgCl_2$, 1.5 mM. Four 10-fold serial dilutions of cDNA from RT reaction and 2 U *Taq* polymerase (Gibco BRL) were used for each PCR. PCR was performed as follows: initial denaturation step at 94°C for 4 min followed by 35 reamplification rounds (94°C for 30 s, 60°C for 30 s, 72°C for 60 s).

Quantitative real-time RT-PCR. Total RNA was extracted using an RNAqueous kit (Ambion, AMS Biotechnology Ltd.) from the selected brain regions of control or cocaine-treated rats. RT was performed essentially as described (Liang and Pardee, 1992; Brenz Verca *et al.*, 1998). Primers for quantitative PCR were designed using PrimerExpress software (PE Applied Biosystems, Foster City, CA) based on CD81 GenBank sequences for rat (TAPA-1). The 28S ribosomal RNA (rRNA) was used as an endogenous control. The forward and reverse primers for CD81 were, respectively, 5′-GGC TAG GCT CCA ACC CTT CT-3′ and 5′-GAC TGC GGT GTG GCT ATT GAG-3′. Primers for the control 28S rRNA were 5′-CAG CGA AAC CAC AGC CAA G-3′ and 5′-CTT CTT TCC CCG CTG ATT CC-3′. Up to five independent real-time PCRs were carried out on an iCycler (Bio-Rad). The measure of SYBR green fluorescence was used for quantification of PCR product. The reaction was carried out in 50 μl final volume containing 25 μl SYBR Green PCR Master Mix (PE Applied Biosystems), 300–900 nM each primer, and variable amount of cDNA template. PCR was performed as follows: initial denaturation step at 94°C for 10 min followed by 40 annealing–extension cycles (94°C for 15 s, 60°C for 60 s). The relative fluorescence at the threshold cycle was used to calculate the relative expression of CD81, using the 28S rRNA as endogenous control.

In situ hybridization. *In situ* hybridization was performed essentially as described (Braissant and Wahli, 1998). Briefly, DIG- or fluorescein-labeled sense and antisense probes were produced using T3, T7, or SP6 promoters from plasmids containing the clones of in-

terest. Before the *in vitro* transcription reaction the plasmids were digested nearest the insert with suitable restriction enzymes to prevent the contamination of probe with vector sequences. The mouse MD3 (CD81) complete cds clone in pCRII vector, the rat CART complete cds clone corresponding to the short form of CART peptide in pBSII SK(+) vector, and the rat TH partial cDNA clone (≈1500 bp) in pSPT18 vector were kindly provided by E. Tsitsikov (Harvard), S. Hastrup (Novo Nordisk, Denmark), and N. Faucon Biquet (CNRS, Paris, France), respectively. TH probe was hydrolyzed for 3 min in 0.2 M carbonate buffer (80 mM $NaHCO_3$/120 mM Na_2CO_3, pH 10.2) at 60°C to the final fragment length of ≈500 bp. Serial dilutions of riboprobes were spotted on positively charged nylon membrane together with serial dilutions of DIG-labeled antisense neo-RNA (Boehringer Mannheim) of known concentration as standard, to estimate the efficiency of the labeling reaction. In addition, probes were loaded on 2% agarose gel to check for RNA size and integrity.

Rat brains were rapidly frozen in isopentane upon extraction and stored dry at −80°C. Twenty-five-micrometer sections were cut on a cryostat, then dried at room temperature, and stored at −20°C. For *in situ* hybridization, sections were postfixed for 10 min in 4% paraformaldehyde in phosphate-buffered saline (PBS), incubated 2 × 15 min in PBS containing 0.1% active diethylpyrocarbonate, rinsed briefly in 5× saline sodium citrate (SSC), and prehybridized for 1–2 h in hybridization buffer containing 50% formamide, 5× SSC, and 40 μg/ml salmon sperm DNA at 58–61°C. Hybridization was then carried out in the same buffer and at the same temperature with 50–100 ng/ml DIG- or fluorescein-labeled probe overnight. After hybridization sections were washed for 30 min at room temperature, then for 1 h at 65°C in 2× SSC, and for 1 h at 65°C in 0.1× SSC. For *in situ* hybridization with TH probe, the additional step of RNase treatment (30 μg/ml at 30°C) after hybridization was necessary to remove nonspecific staining. After washing the detection was performed with anti-DIG or anti-fluorescein Abs coupled to alkaline phosphatase and color development with NBT/BCIP substrate (all reagents from Boehringer Mannheim). Nonspecific staining was then removed by washing for 1 h in 95% EtOH. The images of entire sections were digitalized using videocamera and Image Store 5000 hardware (UVP). Photomicrographs were prepared by capturing the images with a Spot Camera (Diagnostic Instruments, Inc.) on a Zeiss Axiophot light microscope.

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ANNEX III

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MOLECULAR BRAIN RESEARCH

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Research report

Altered sensitivity of CD81-deficient mice to neurobehavioral effects of cocaine

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Abstract

CD81, also known as target of the antiproliferative antibody, is known to be expressed in astrocytes and involved in cell adhesion and, recently, we demonstrated its induction exclusively in the accumbens following cocaine. In the present study, the sensitivity of CD81-deficient mice to behavioral effects of cocaine was evaluated. It was found that CD81-deficient mice exhibited altered sensitivity to cocaine as assessed in the place preference conditioning paradigm and locomotor activity. This deficit in place preference conditioning was not accompanied by a deficit in acquisition or retention of water maze behavior. In addition, CD81 knockout mice exhibited higher levels of nucleus accumbens dopamine as compared to their controls. These observations are discussed in the context of the role of CD81 in cocaine-mediated behaviors.

Theme: Neural basis of behavior

Topic: Drugs of abuse: cocaine

Keywords: CD81; Cocaine; CD81-knockout mice; Water maze; Place preference; Accumbens dopamine; Activity

1. Introduction

CD81, also known as TAPA-1 (target of the antiproliferative antibody) is a member of the tetramembrane spanning family of proteins, and is involved in signal transduction, cell–cell adhesion, and cellular activation or development [5,9,10]. CD81 is a component of the signal transducing complex found in B lymphocytes [11,17] and has been shown to play a role in the development of T cells [2]. In the CNS, this protein is expressed primarily by the ependyma, choroid plexus, astrocytes, and oligodendrocytes; however, there remains a possibility that neurons may also express this protein [16]. CD81 is expressed at points of glial cell contacts and is believed to contribute to their stabilization. Exposure of glial cell cultures to the anti-TAPA antibody AMP1 reduces their mitotic activity [5]. Its expression is dramatically upregulated following physical injury, suggesting that this protein may play a prominent role in the response of astrocytes to injury and in glial scar formation [5].

In a series of screening studies intended to identify molecular cues induced by psychomotor stimulants, we observed an increased expression of the CD81 gene 24 h after cocaine administration to rats. Of interest, this induction of CD81 by cocaine was observed only in the accumbens [3]. Cocaine is a dopaminergic agonist which exerts its stimulant effects by blocking dopamine reuptake following its release [14]. Cocaine is self-administered by

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humans and other animals [1,19] and is a major drug of abuse. The primary site of action in regard to the reinforcing action of cocaine is the nucleus accumbens [4,13–15]. With its repeated administration, both tolerance and sensitization develop to many of the behavioral effects of cocaine and such changes may mediate the symptoms of 'craving' and 'relapse' which renders cocaine abuse a tenacious problem [6,7]. Although the molecular mechanism mediating the development of this tolerance and/or sensitization remains elusive, it is thought that cocaine may induce changes in the expression of membrane proteins on its target cells and that these proteins, in turn, alter neuronal plasticity in response to the drug [7,8].

The objective of our initial study [3] was to identify molecular cues induced by the acute administration of cocaine. Having identified CD81 as such a cue, we then assessed the neurochemical and behavioral effects of cocaine administered to CD81-deficient mice. The behavioral paradigms employed for this later objective were locomotor activity (used to assess the stimulant properties of the drug) and place preference conditioning (used to assess the reinforcing efficacy of the drug). Finally, since deficits in place preference conditioning were observed, a water maze task was employed to determine if these deficits were consequent to non-specific impairments in the behavioral ability of CD81-deficient mice.

2. Materials and methods

2.1. Subjects

Eighteen male and 18 female CD81-deficient mice with a C57BL/6 background (initially obtained from E. Tsitsikov; see [18]) and their controls, 18 male and 18 female C57BL/6 mice (Charles River Laboratories, Raleigh, NC, USA), approximately 3-months-old and weighing 28–35 g at the beginning of testing were used. The CD81-deficient mice were of the sixth generation of breeding into the C57BL/6 background. Animals were housed individually in pan cages containing woodchip bedding in a colony room with a 12 h light/dark cycle (lights on at 7.00) and were given free access to food and water. Experimentation took place during the light phase of the cycle and was conducted at the same time each day. Six mice of each strain and sex were used in the following studies.

2.2. Place preference

Place preference tests were performed in an opaque Plexiglas apparatus (20×20×30 cm) divided into four separate compartments (12×8×10 cm) with a central neutral compartment. The neutral compartment was used as the place of introducing the animal on baseline and test days. The neutral compartment had a black Plexiglas floor and clear Plexiglas walls. Three of the four chambers were lined with absorbent paper while the fourth chamber was designated as the bare compartment chamber. After each mouse, compartments were thoroughly cleaned and paper lining the floor was changed.

The place preference study consisted of three phases: baseline, conditioning, and testing. All phases occurred at the same time each day and in an isolated room. In the baseline phase (3 days), subjects were placed in the neutral compartment and were allowed free access to the entire apparatus for 15 min. The amount of time spent in each chamber was recorded. During the conditioning phase (12 days), subjects were given an i.p. injection of either cocaine HCl (10 mg/kg) or an equal volume of saline, on an alternating schedule (six cocaine and six saline). Five minutes after injection, animals were locked into a specific chamber for 15 min. If receiving cocaine, animals were placed in the bare chamber while, after saline, animals were placed into one of the three lined chambers. For the post-conditioning phase, subjects were tested for their preference in a drug-free state. Each mouse was placed in the neutral compartment and was allowed free access to the entire chamber for 15 min. The amount of time spent in each chamber was then recorded and presented as the percentage of time spent in the bare 'cocaine-associated' chamber divided by the amount of time spent in the lined 'saline-associated' chambers.

2.3. Activity

The locomotor activity test apparatus consisted of a 43×43 cm Plexiglas chamber, surrounded by a 12×12 grid of photo cells. The chamber was thoroughly cleaned after testing each mouse. On separate days, animals received i.p. injections of saline or cocaine HCl (0.3, 1, 3 or 10 mg/kg) 15 min prior to placement in the chamber for 10 min. Horizontal motor activity was recorded by photocell beams.

2.4. Water maze

The water maze (61 cm diameter and 28 cm high) was filled with opaque, room-temperature water to a depth of 15 cm and was located in a test room measuring 3.2×2.1 m. A 7.5 cm diameter platform was submerged 2.5 cm below the water surface and was located 18 cm from the side. Trials were initiated by placing a mouse in one quadrant of the maze and allowing it to swim to the platform with a maximum 60 s trial. Four trials were conducted each day, each starting from a different quadrant. Trials were conducted for 5 successive training days, followed by 3 days without training and concluded by 2 successive days of retention trials conducted in the same manner.

2.5. Neurochemistry

Upon the completion of behavioral tests, animals were sacrificed, striatum and nucleus accumbens dissected and stored in liquid nitrogen until assayed by HPLC (Bioanalytical Systems, West Lafayette, IN). Tissue was homogenized in 0.3 ml of 0.4 N perchloric acid with 0.1 mM ethylenediaminetetra-acetic acid (EDTA) added to inhibit biochemical degeneration. Samples were centrifuged at 20,000 *g* for 20 min at 4°C and supernatant assayed for dopamine, serotonin, and their metabolites, dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), and 5-hydroxyindoleacetic acid (5-HIAA). The mobile phase consisted of 0.1375 M sodium phosphate (dibasic), 0.0625 M citric acid, 5.0 mg EDTA and 14% methanol with a flow rate of 0.7 ml/min.

3. Results

3.1. Place preference

On the last baseline day, there were no difference in preference for lined or bare chambers between male or female C57BL/6 versus CD81-deficient mice. After conditioning, both male (Fig. 1A) and female C57BL/6 (Fig. 2A) mice spent more time in the bare, cocaine-paired chamber than in the lined, saline chamber. These differences were statistically significant and were seen on all three test days (see figure legend for *F*-values).

Likewise, female CD81-deficient mice (Fig. 2B) spent significantly more time in the bare, cocaine-paired chamber on the first day after conditioning, but this effect disappeared by the second day of testing. Finally, after conditioning, male CD81-deficient mice spent approximately equal time in the lined compared to the bare chamber (Fig. 1B).

3.2. Activity

Male C57BL/6 mice were more active than male CD81-deficient mice following 0.1 mg/kg of cocaine (Fig. 3A). Cocaine at 10 mg/kg increased the activity of the CD81-deficient mice but not the C57BL/6 mice. Female C57BL/6 mice were more active than female CD81-deficient mice under baseline conditions as well as following 0.1 and 3 mg/kg cocaine (Fig. 3B); however, this effect reversed itself following 1 mg/kg cocaine.

3.3. Water maze

There was a significant improvement in performance over the five training days for male (Fig. 4A) and female (Fig. 4B) C57BL/6 and knockout mice but there were no differences between these strains for either sex. In addition, both strains exhibited full retention after a 3 day delay, again, with no differences between strains.

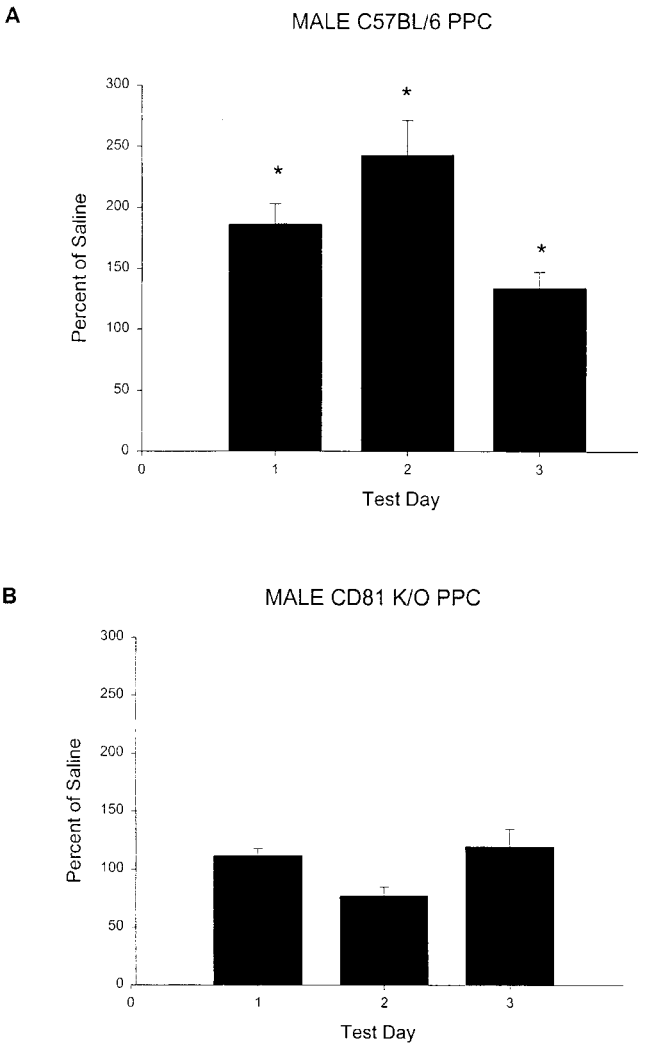

Fig. 1. Place preference behavior for male mice during 3 days of testing. Test days followed 12 days of conditioning during which cocaine was paired with the 'bare' chamber. Data represent percentage of time mice spent in the bare 'cocaine-associated' chamber divided by the amount of time spent in the lined 'saline-assoicated' chambers. (A) C57BL/6: ($F_{5,25}$=32.92; P=0.0001); (B) CD-81 deficient: P>0.05. *significantly greater than 100%, Fisher's LSD test; P<0.05.

3.4. Neurochemistry

In the striatum, there were no significant differences between the male C57BL/6 and CD81-deficient mice in the levels of dopamine, serotonin, DOPAC, HVA or 5-HIAA. In the accumbens, there was a significant increase in the concentration of dopamine in the CD81 knockout mice (t_{10}=2.4; P=0.03) along with a significant increase in the concentration of DOPAC (t_{10}=3.12; P=0.01) (Table 1). In females, there were no significant differences in the striatum or accumbens between the C57BL/6 and CD81-

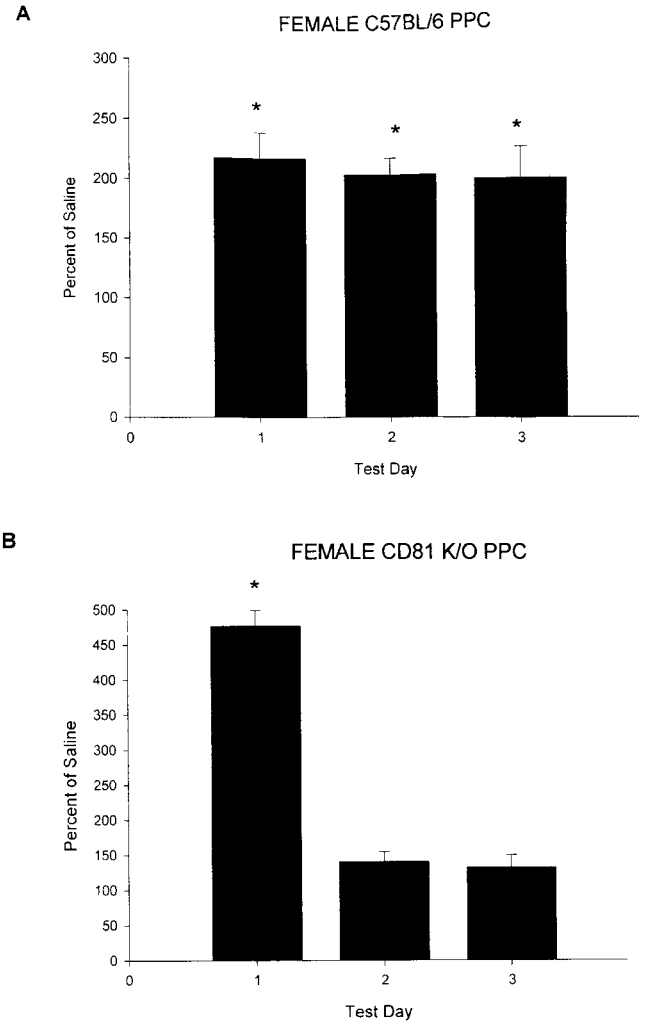

Fig. 2. Place preference behavior for female mice during 3 days of testing. The test days followed 12 days of conditioning during which cocaine was paired with the 'bare' chamber. Data represent the percentage of time mice spent in the bare 'cocaine-associated' chamber divided by the amount of time spent in the lined 'saline-associated' chambers. (A) C57BL/6: ($F_{5,25}$=5.86; P=0.001); (B) CD-81 deficient: ($F_{5,25}$=3.23;P=0.02. *significantly greater than 100%, Fisher's LSD test; P<0.05.

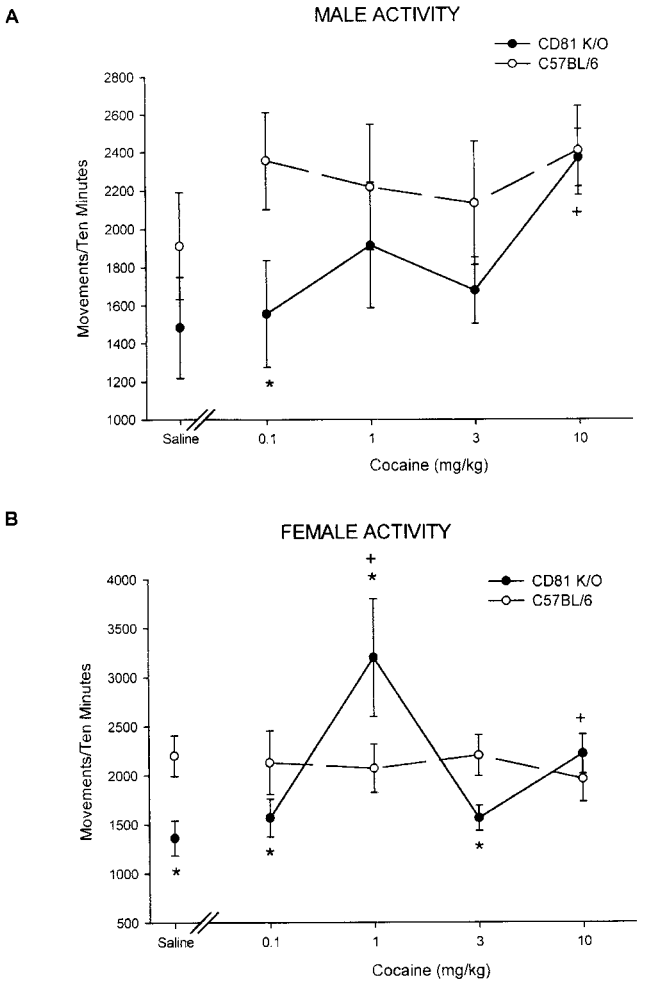

Fig. 3. Activity levels of male (A) and female (B) C57BL/6 and CD81-deficient mice under baseline (saline) conditions or following the administration of cocaine. Male ($F_{4,4}$=3.6; P=0.013) and female ($F_{4,4}$= 7.4; P=0.0001) C57BL/6 mice were significantly more active than their CD81 K/O counterparts at each dose and, for females, there was a significant dose by strain interaction ($F_{4,40}$=10.3; P=0.0001). *significantly different from C57BL/6; P<0.05, Fisher's LSD test. $^{+}$significantly different from saline; P<0.05, Fisher's LSD test.

deficient mice in the levels of dopamine, serotonin, DOPAC, HVA or 5-HIAA.

4. Discussion

4.1. Cocaine induces CD81 expression in rat accumbens

Cocaine is considered to be a major drug of abuse world-wide. Its reinforcing effects are mediated in a large part through the dopaminergic neurons in the nucleus accumbens. With its repeated use, molecular changes occur in these cells that predispose the individual to continue to crave cocaine as well as rendering this individual more prone to relapse following cocaine withdrawal [7]. In a previous study, we demonstrated that the administration of a high-dose regimen of cocaine to rats caused an induction of the CD81 gene. This gene was expressed 24 h after the last cocaine injection and, more important, was observed exclusively in the nucleus accumbens [3]. Furthermore, although it was initially thought that central CD81 expression was restricted to glia cells [5,16], we demonstrated its presence in a variety of neurons, especially those associated with hypothalamic regulation of cardiovascular function [3]. However, the fact that CD81 expression was selectively induced in the nucleus accumbens by cocaine

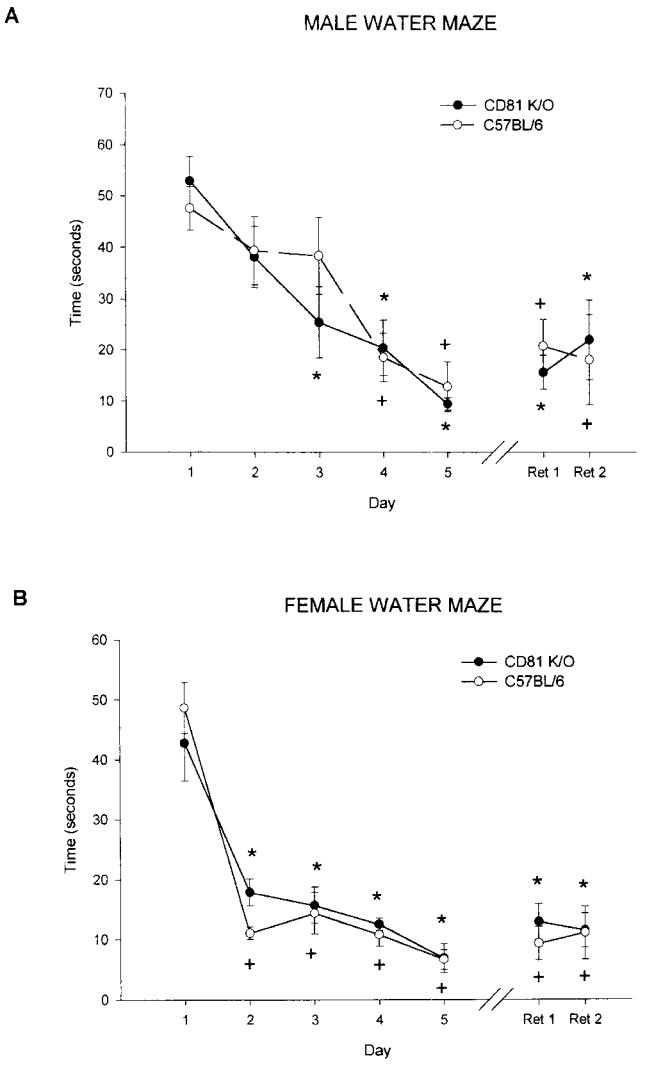

Fig. 4. Water maze data for male (A) and female (B) C57BL/6 and CD81-deficient mice. All groups showed a significant improvement over five training days (males: $F_{6,60}=13.09$; $P=0.0001$; females: $F_{6,60}=37.18$; $P=0.0001$). There was no significant deficit in retention for any of the groups and there were no significant differences between strains for either sex. *significantly different from C57BL/6; $P<0.05$, Fisher's LSD test. $^{+}$significantly different from day 1; $P<0.05$, Fisher's LSD test.

prompted us to evaluate the reinforcing efficacy of this widely-abused drug in CD81-deficient mice.

4.2. Diminished sensitivity to cocaine-induced place preference in CD81-deficient mice

Male and female C57BL/6 mice exhibited a strong place preference conditioning clearly preferring the bare chamber following cocaine conditioning. In contrast, male CD81-deficient mice exhibited no place preference conditioning whatsoever, while the females exhibited preference for the bare chamber only during the first day of testing; thereafter, they also exhibited no preference for the cocaine-paired chamber. One interpretation of these data is that the CD81-deficient mice are less sensitive to the reinforcing effects of cocaine.

Table 1
Transmitter and metabolite levels

	Dopamine	DOPAC	HVA	Serotonin	5-HIAA
Males					
Striatum					
CD81 K/O	6.61	0.579	0.821	0.461	0.276
	(1.296)	(0.118)	(0.165)	(0.087)	(0.05)
C57BL/6	6.415	0.557	0.757	0.45	0.25
	(0.827)	(0.07)	(0.078)	(0.015)	(0.009)
Accumbens					
CD81 K/O	4.42*	0.64*	0.76	0.714	0.364
	(0.832)	(0.058)	(0.087)	(0.083)	(0.029)
C57BL/6	1.99	0.375	0.614	0.673	0.279
	(0.564)	(0.062)	(0.149)	(0.095)	(0.038)
Females					
Striatum					
CD81 K/O	6.562	0.772	1.053	0.581	0.384
	(0.767)	(0.069)	(0.121)	(0.052)	(0.013)
C57BL/6	4.779	0.632	0.989	0.49	0.314
	(0.491)	(0.052)	(0.121)	(0.036)	(0.015)
Accumbens					
CD81 K/O	1.593	0.276	0.298	0.687	0.327
	(0.377)	(0.051)	(0.059)	(0.038)	(0.05)
C57BL/6	1.921	0.239	0.262	0.733	0.222
	(0.244)	(0.025)	(0.014)	(0.015)	(0.008)

Values reported are in μg/g tissues±(S.E.M.). *significantly different from C57BL/6.

However, an alternative interpretation might be that the CD81-deficient mice have a poor retention of learned tasks. This interpretation can be ruled out on the basis of the results obtained on the water maze retention task. In this study, CD81-deficient mice exhibited nearly identical acquisition and retention curves as compared to their C57BL/6 controls. Thus, it appears that this deficit in place preference performance can not be attributed to a non-specific disruption of learning or memory processes.

An alternative interpretation of the deficit in place preference performance exhibited by the CD81-deficient mice might be that C57BL/6 mice were used as controls in place of wild-type littermates. However, since the CD81-deficient mice have been backcrossed into the C57BL/6 strain for six generations, the possibility of difference in place preference but not water maze performance being due to strain differences is remote. Finally, the molecular corroboration of these data (i.e. CD81 induction in only the accumbens following cocaine [3]) point convincingly to this gene mutation being involved in the altered sensitivity to the reinforcing efficacy of cocaine. It still remains to be determined if the deficit in place preference

performance exhibited by the CD81-deficient mice is restricted to cocaine or is evident for other drugs of abuse.

4.3. Activity levels in CD81-deficient mice following cocaine

Still another interpretation of the above place preference data is that the CD81-deficient mice may exhibit a different sensitivity to the stimulant effects of cocaine. In the activity task, the C57BL/6 mice were generally more active than the CD81-deficient mice under baseline conditions as well as following the administration of cocaine. This was the case following the administration of all doses of cocaine to males and females with the notable exception of the heightened response of the CD81-deficient females to the 1.0 mg/kg dose. Thus, since the activity measure revealed that CD81-deficient mice have an altered sensitivity to cocaine, it may be possible that the deficit in place preference conditioning reflects an altered sensitivity to the reinforcing effects of the drug.

4.4. Increased accumbens dopamine in CD81-deficient mice

Finally, neurochemical analysis of the CD81-deficient mice revealed that levels of dopamine and its metabolite, DOPAC, were increased in the nucleus accumbens of male, but not female mice. The exact mechanism through which CD81 (or its absence) may alter neural activity remains uncertain. However, we have recently demonstrated that CD81 is not restricted to glial cells but is also present on neurons [3].

The increased concentration of dopamine in the accumbens together with the reduction in place preference conditioning may indicate that sensitivity of these mice to cocaine was increased to a point where it was no longer reinforcing. In support of this interpretation it has been shown that high doses of cocaine fail to induce place preference conditioning [12]. This may indicate that the dose used in the place preference conditioning study (in combination with the excessive amount of accumbens dopamine) was too high for the CD81-deficient mice. Though speculative, this interpretation may help explain the observed differences between males and females in place preference conditioning. That is, males with the higher accumbens dopamine did not show any place preference following cocaine whereas females with near normal levels of dopamine did exhibit a transient place preference for the cocaine-associated chamber.

In summary, CD81-deficient mice were found to have an altered sensitivity to cocaine in place preference conditioning and locomotor activity and to have higher levels of dopamine and DOPAC in their nucleus accumbens. They did not show any deficit in water maze behavior. Taken together with our previous report [3], these data may indicate that this protein is involved in mediating the acute and/or long-term neural responsiveness to cocaine.

Acknowledgements

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Table 1: Transmitter and Metabolite Levels

MALES

STRIATUM

	DOPAMINE	DOPAC	HVA	SEROTONIN	5-HIAA
CD81 K/O	6.61 (1.296)	0.579 (0.118)	0.821 (0.165)	0.461 (0.087)	0.276 (0.05)
C57BL/6	6.415 (0.827)	0.557 (0.07)	0.757 (0.078)	0.45 (0.015)	0.25 (0.009)

ACCUMBENS

	DOPAMINE	DOPAC	HVA	SEROTONIN	5-HIAA
CD81 K/O	4.42* (0.832)	0.64* (0.058)	0.76 (0.087)	0.714 (0.083)	0.364 (0.029)
C57BL/6	1.99 (0.564)	0.375 (0.062)	0.614 (0.149)	0.673 (0.095)	0.279 (0.038)

FEMALES

STRIATUM

	DOPAMINE	DOPAC	HVA	SEROTONIN	5-HIAA
CD81 K/O	6.562 (0.767)	0.772 (0.069)	1.053 (0.121)	0.581 (0.052)	0.384 (0.013)
C57BL/6	4.779 (0.491)	0.632 (0.052)	0.989 (0.121)	0.49 (0.036)	0.314 (0.015)

ACCUMBENS

	DOPAMINE	DOPAC	HVA	SEROTONIN	5-HIAA
CD81 K/O	1.593 (0.377)	0.276 (0.051)	0.298 (0.059)	0.687 (0.038)	0.327 (0.05)
C57BL/6	1.921 (0.244)	0.239 (0.025)	0.262 (0.014)	0.733 (0.015)	0.222 (0.008)

Values reported are in μg/g tissues ± (SEM)
* = significantly different from C57BL/6

Annex IV

Cocaine-induced expression of γ-synuclein in the adult rat brain after acute drug withdrawal

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Running title: ***"Cocaine-induced γ-Synuclein in the adult rat brain"***

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INTRODUCTION

Synucleins are small (120-140 amino acids) proteins sharing structural resemblance with apolipoproteins (Clayton and George, 1999). Four members, α-, β-, γ-synucleins and synoretin, have been described in different organisms, including humans, rodents, fish and birds. α- and β-synucleins are predominantly presynaptic proteins (Iwai et al., 1995). β-synuclein is highly homologous to α-synuclein, but it lacks 11 amino acids in the region corresponding to the NAC peptide (Lavedan, 1998). The most distinct member of the family, γ-synuclein/persyn, lacks a conserved C terminal domain of α- and β-synucleins and displays different spatial and temporal expression, being distributed diffusely throughout the cell body and axons, at least in peripheral neurons (Buchman et al., 1998). Recently the fourth member, synoretin, has been identified in retinal cells and in the brain where it plays a role in the regulation of the Elk1 pathway (Surguchov et al., 1999).

Synucleins have attracted much attention because of their involvement in several neurodegenerative disorders. In Alzheimer disease (AD) α-synuclein/NACP (non-Aβ component of amyloid precursor) is the precursor of the NAC peptide, the intrinsic component of amyloid plaques. In Parkinson's disease (PD) two point mutations in α-synuclein are genetically linked to the disorder (Polymeropoulos et al., 1997; Lavedan, 1998) and the protein forms the major fibrillary component of Lewy bodies (Clayton and George, 1999). Mutations in the α-synuclein may change the secondary structure of otherwise unfolded protein promoting the formation of aggregates. Interestingly, one of the point mutations is naturally present in rodent orthologues of α-synuclein, as well as in human γ-synuclein. β- and γ-synucleins were also observed in axons of patients with PD or Lewy body dementia (Galvin et al., 1999). In addition γ-synuclein stimulates breast cancer invasion and metastasis (Jia et al., 1999). Synucleins are mainly expressed in neurons, but they are also a component of glial cytoplasmic inclusions which are characteristic of multiple system atrophy and amyotrophic lateral sclerosis (Lavedan, 1998) and are abundant in the brain stem, basal ganglia, and cortical white matter (Gai et al., 1999).

Several studies indicate a role of synucleins in synaptic plasticity. Synucleins interact with intracellular signal transduction cascades. They contain KTKEGV repeat sequences homologous to Rho-GTPases, and play a role in neuritogenesis (Mackay et al., 1995). They are powerful inhibitors of phospholipase D2 (Jenco et al., 1998), whose function in cytoskeletal organization is well established (Colley et al., 1997). In addition γ-synuclein/persyn, influence neurofilament network integrity by increasing the susceptibility of NF-H to calcium-dependent proteases (Buchman et al., 1998). The expression of synucleins is up-regulated in the substantia nigra (SN), the cortex and the hippocampus during the postnatal period, when synaptic contacts develop (Coyle, 1977; Kholodilov et al., 1999a; Petersen et al., 1999). In the SN synuclein mRNA is also up-regulated upon injury, the overexpression being exclusively restricted to surviving, non-apoptotic, neurons (Kholodilov et al., 1999a), probably as a major compensatory reaction. The down-regulation of α-synuclein was also described, e.g. upon intrastriatal injection of 6-hydroxydopamine as dopamine neurons die (Kholodilov et al., 1999b) or in patients with early-onset of PD (Neystat et al., 1999).

In a screening for genes differentially expressed after cocaine withdrawal we found that γ-synuclein was a major up-regulated candidate. For this reason we investigated in more detail the distribution of α-and γ-synucleins in the adult rat brain and present here a distinct pattern of expression for each protein.

METHODS

Drug treatment. Eight week old, male Sprague-Dawley rats (Taconic Farms, Germantown, NY) were injected subcutaneously with 30 mg/kg cocaine-HCl (Sigma Chemical Co., St Louis) every 2hrs for 4 injections. Control animals received the saline injections under the same schedule. The rats were sacrificed 24 hours after the last injection, and tegmental area was dissected out and used for mRNA isolation.

RNA isolation and Differential Display analysis. Total RNA was extracted using an RNAqueous kit (Ambion™, AMS Biotechnology Ltd., Lugano, Switzerland) from the tegmental tissue of saline- or cocaine-treated rats. Differential display was then performed as described (Brenz Verca et al., 1998). Briefly, 1 µg total RNA was reverse transcribed with modified oligo(dT) primer sets. Reaction conditions were set according to Liang & Pardee (Liang and Pardee, 1992). Subsequent PCR was performed with the same sets of oligo(dT) primers in addition to arbitrary primers XAp1 to XAp20. PCR was performed as follows: five cycles at low stringency (94°C, 60"; 40°C, 120"; 72°C, 60") followed by 35 cycles at high stringency (94°C, 45"; 56°C, 60"; 72°C, 60"). A typical PCR reaction was carried out in 20 µl final volume with the following reagents: 2 µM dNTPs, 1 µM 3'-anchoring primer, 1µM arbitrary primer, 1 mM $MgCl_2$, 1/50 of the cDNA obtained from a reverse transcription, 1-2 µCi [^{32}P]-dATP at 3000 Ci/mmol (Hartmann Analytic) and 1.5 U Taq polymerase (Gibco BRL) were used for each PCR. Reaction products were resolved on 6% denaturing sequencing gels, which were dried and exposed to Fuji X-ray film overnight. Excised gel slices were rehydrated in 100 µl 1x TE at 80 ˚C during 10 min. Reamplifications were carried out in 40 µl total volume with the following reagents: 250 µM dNTPs, 1µM reamplification primers, 2.5 mM $MgCl_2$, 1.5 U/reaction Taq DNA polymerase, 5 µl cDNA eluate/reaction. Cycling conditions were five cycles (94°C, 45"; 40°C, 60"; 72°C, 60") followed by

35 cycles at high stringency (94°C, 60"; 62°C, 60"; 72°C, 60") and a 15 min extension step at 72 ˚C. PCR products were cloned into pBluescript KS+ vector. Clones were subjected to analytical restriction analysis with RsaI and the prominent sequences were sequenced by Microsynth GmbH.

In situ hybridization. In situ hybridization was performed essentially as described (Braissant and Wahli, 1998). Briefly, digoxigenin (DIG)- or fluorescein-labeled sense and antisense probes were produced using T3, T7 or SP6 promoters from plasmids containing the clones of interest. Before the *in vitro* transcription reaction the plasmids were digested nearest the insert with suitable restriction enzymes to prevent the contamination of probe with vector sequences. The rat synuclein-1 (α-synuclein) complete cds clone in pGEM-T vector, the rat sensory synuclein (γ-synuclein) clone in pT7T3Pac vector and the tyrosine hydroxylase (TH) partial cDNA clone (≈1500bp) in pSPT18 were kindly provided by N.Kholodilov (Columbia University), K.Scott (Iowa University) and N. Faucon Biquet (CNRS, Paris, France) respectively. TH probe was hydrolyzed for 3 min in 0.2M carbonate buffer (80mM $NaHCO_3$/120mM Na_2CO_3, pH 10.2) at 60°C to the final fragment length of ≈500bp. Serial dilutions of riboprobes were spotted on positively charged nylon membrane together with serial dilutions of DIG-labeled antisense neo-RNA (Boehringer Mannheim) of known concentration as standard, to estimate the efficiency of the labeling reaction. In addition, probes were loaded on 2% agarose gel to check for RNA size and integrity. Rat brains were rapidly frozen in isopentane upon extraction and stored dry at –80°C. 25 µm sections were cut on a cryostat, then dried at RT and stored at –20°C. For *in situ* hybridization, sections were postfixed for 10 min in 4% paraformaldehyde in PBS, incubated 2x15 min in PBS containing 0.1% active diethylpyrocarbonate, rinsed briefly in 5x saline sodium citrate (SSC) and pre-hybridized for 1-2 hrs in hybridization buffer containing 50% formamide, 5xSSC, and 40 µg/ml salmon sperm DNA at 58-61°C. Hybridization was then carried out in the same buffer and at the same temperature with 50-100 ng/ml DIG- or fluorescein-labeled probe overnight. After hybridization sections were washed for 30 min at RT, then for 1 hr at 65°C in 2xSSC, and for 1 hr at 65°C in 0.1xSSC. For *in situ* hybridization with TH probe, the additional step of RNase treatment (30 µg/ml at 30°C) after hybridization was necessary to remove unspecific staining. After washing the detection was performed with anti-DIG or anti-fluorescein antibodies coupled to alkaline phosphatase and color development with NBT/BCIP substrate (all reagents from Boehringer Mannheim). Non-specific staining was then washed out with 95% EtOH for 1 hr. The images of entire sections were digitalised using videocamera and Image Store 5000 hardware (UVP). Photomicrographs were prepared by capturing the images with a Axiocam on a Zeiss Axiophot light microscope, labeling was performed using Adobe Photoshop (Adobe Systems) and Freehand (Macromedia) software.

RESULTS

Induction of γ-synuclein after cocaine treatment by Differential Display

To examine the changes in gene expression in the dopaminergic brain system upon acute cocaine treatment, we used a modified protocol for differential display (Brenz Verca et al., 1998). Patterns of expression were compared for mRNAs isolated from tegmental area of saline- or cocaine-treated rats. Differential display screening was performed in duplicate from two independent reverse transcriptions and resulted in 15 candidates with up- or down-regulation in tegmentum following cocaine. All candidate tags were cut out from differential display gel, and cloned. Each fragment was checked for sequence homogeneity by means of analytical restriction of about 10 independent clones with RsaI. The representatives of the most abundant restriction patterns were processed to further analysis, including sequencing and search for homology in Genbank database. The validity of the differential display screening was supported through the finding of genes known to be modified by cocaine treatment. For example, the NADH dehydrogenase subunit 6, an mRNA product of the mitochondrial genome, was up-regulated, in accordance with the literature (Couceyro et al., 1997). Among the candidates with altered expression after cocaine treatment a gene with 100% homology with sensory neuron synuclein (rat homologue of γ-synuclein) (Fig.1) displayed a pattern of "all-or-none" up-regulation in the tegmental area. The homology was found in the 3' region of mRNA sequence. This region is not included in the high homology region, defining the synuclein family (Fig.2), thus we could un-equivocally identify our message as γ-synuclein.

Expression of α- and γ-synuclein in the rat brain

In situ hybridization was performed with a γ-synuclein probe on rat brain coronal sections (Fig.3 and Table 1). The probe was prepared from the γ-synuclein clone in a region with no homology to other members of the synuclein family. γ-synuclein is homologous to α-synuclein, a protein involved in the pathogenesis of several neurodegenerative diseases. We have compared the distribution of both mRNAs with a view to better understand their respective functions. The distribution of α-synuclein message has already been described elsewhere (Maroteaux and Scheller, 1991) and confirmed in our experiments. In the following section we will focus on differential expression of the two proteins. In general α-synuclein displays a very characteristic pattern of expression, confined to specific nuclei, whereas γ-synuclein is more widely expressed throughout the brain.

Cerebral cortex

In the cerebral cortex, the expression of α-synuclein is confined primarily to layers II and V of cerebral cortex (Fig.3, 4A) with the lowest expression in parietal and highest expression in insular and piriform cortices respectively. In contrast γ-synuclein is distributed more

uniformly within the different cortical layers and is expressed at low levels, particularly within layer V. Both proteins display a very complementary pattern of expression throughout most of the cortex.

Ependyma and circumventricular organs

Ependymal cells lining the ventricles as well as those of the choroid plexus were positive for γ-, but not for α-synuclein. γ-synuclein is a strong marker of the ependymal layer of the ventricles. The expression is very dense and confined to virtually all ependymal cells (Fig.4D). We found high expression of γ-synuclein in both the olfactory (Fig.3B) and the lateral (Fig.3D) ventricles and also a dense labeling in the subfornical organ (Fig.4C).

Olfactory System and Basal Ganglia

α-synuclein was very strongly expressed in almost all olfactory areas, including the anterior olfactory nucleus (Fig.3C, 5A), the tenia tecta, the piriform cortex (Fig.3C,E, 5C) and the nucleus of the lateral olfactory tract (Fig.5E). γ-synuclein was present in all these regions, but to a much lesser extent (Fig.3D, 5B,F). On the other hand the semilunar nucleus was positive exclusively for γ-synuclein (data not shown). γ-synuclein was highly expressed in the olfactory tubercle (Fig.3D), the ventral pallidum (Fig.5B) and the caudate-putamen (Fig.3F) and to a lesser extent the ventral striatum (nucleus accumbens (NAcc)). In addition, γ-synuclein is a suitable marker for the matrix compartments of the caudate-putamen (Fig.5D). In contrast α-synuclein was only faintly expressed in the basal ganglia.

Clearly α-synuclein is expressed mostly in olfactory areas and γ-synuclein mostly in the basal ganglia. α-synuclein labeling clearly delimits the border between the piriform cortex and the olfactory tubercle (Fig.5C), as well as between the anterior olfactory nucleus and the NAcc (Fig.3C). By contrast expression of γ-synuclein in these regions is relatively homogeneous (Fig.3D), because of its lower expression in olfactory areas (anterior olfactory nucleus and piriform cortex) and its higher expression in basal ganglia (olfactory tubercle and NAcc).

Septum, extended amygdala and hippocampus

All septal nuclei were positive for γ-synuclein mRNA (Table 1 and for example Fig.3F). The labeling was very dense, even if restricted to a limited number of cells, in the nucleus of the horizontal limb of the diagonal band (Fig.6B). α-synuclein was present only in a limited group of septal nuclei (Table 1). In the extended amygdala, γ-synuclein was present non-specifically in all amygdaloid nuclei and more extensively in the bed nucleus of the stria terminalis (Fig.6D), whereas α-synuclein was particularly confined to the basolateral amygdaloid nucleus (Fig.6A), and faintly present elsewhere. Both mRNAs were present in the hippocampus at a relatively high level.

Thalamus and hypothalamus

In the thalamus, α-synuclein was present in a very limited number of nuclei including central medial, paraventricular and rhomboid thalamic nuclei (Fig.3G), with highest level of expression in the paraventricular nucleus (Fig.6C). In contrast γ-synuclein is practically omnipresent in all thalamic regions, including for example the large ventral thalamic nuclei (Fig.3H, 6D).

In the hypothalamus, only the tuberomammillary nucleus was positive for α-synuclein, whereas γ-synuclein was largely presented in this brain region. Fig.7 shows some examples, including the medial preoptic zone (A) and ventromedial and arcuate hypothalamic nuclei (D). Its expression was scattered throughout the lateral hypothalamus, where it was low in the majority of cells, but very high in a subset of large, diffusely distributed neurons (Fig.7B). Only the γ-synuclein message was present in paraventricular and supraoptic hypothalamic nuclei (Fig.3H). Interestingly, the signal in the paraventricular nucleus was mostly confined to subsets of neurons involved in the control of the autonomic nervous system (Fig.7C), including the neurons of the dorsal medial cup and the ventral group, as well as oxytocin-secreting neurons of the medial magnocellular group.

Brain stem

γ-synuclein was present in several brain stem nuclei including the Edinger-Westfal and the red nuclei (Fig.10). A particular distribution of α- and γ-synuclein was found in midbrain dopaminergic nuclei (see below).

Expression of synucleins in dopaminergic nuclei

Both α- and γ-synucleins are related to neurodegenerative disorders affecting the dopaminergic system. In addition, results from the present study show γ-synuclein over-expressed in the tegmentum upon cocaine treatment. In light of these findings, we compared the distribution of synucleins and TH, a marker of catecholaminergic neurons, to assess the expression of synuclein staining in dopaminergic neurons.

Some hypothalamic nuclei are known to contain dopaminergic neurons, e.g. the paraventricular and supraoptic nuclei (Fig.8B,D). γ-synuclein is expressed in both nuclei but not exclusively in dopaminergic cells, as clearly seen when comparing its pattern of expression with the expression of TH on a subsequent section (Fig.8A,C).

The most prominent dopaminergic group of cells is found in the ventral midbrain and comprises the SN and the ventral tegmental area (VTA). Very low levels of expression of synucleins are found in the VTA, but their expression is mostly confined to neurons in the SN (Fig.9). α-synuclein expression is highly correlated with TH, confirming previous findings (Kholodilov et al., 1999a; Abeliovich et al., 2000), whereas γ-synuclein is poorly correlated with TH and is present in addition in several other nuclei, e.g. the red nucleus, devoid of TH and α-synuclein. These results clearly show a distinct pattern of expression for α- and γ-synuclein in relation to the dopaminergic system.

Expression in cocaine-treated brains

The expression of α- and γ-synuclein mRNAs in the tegmental area of saline- and cocaine-treated rat brains have been compared with the expression of TH on subsequent sections, to assess their distribution dopaminergic cell bodies (Fig.10). We could not find obvious differences in the expression of both synucleins, neither in the VTA nor in the SN. However γ-synuclein overexpression upon cocaine is changed in the red nucleus (Fig.10) and its expression is clearly modified at the Edinger-Westfal nucleus. Together these observations confirm our results found by differential display.

DISCUSSION

This study demonstrates that γ-synuclein is up-regulated in the tegmentum after 24 hrs withdrawal from acute cocaine treatment and it compares the brain distribution of α- and γ-synuclein mRNAs. The two members of the synuclein family show a distinct distribution pattern, further suggesting distinct functions for these highly related proteins.

γ-synuclein is implicated in neurodegeneration, e.g. in Parkinson's disease. Its cocaine-induced up-regulation opens new insights on a possible relation between Parkinson's and drug addiction, both of which are related to tegmental dopaminergic neurons. Given the common anatomical substrate for these disorders, attempts to find correlation between their mechanisms of development have been made but were unsuccessful, at least on the symptomatic level. For example, chronic heavy cocaine abuse does not cause parkinsonism (Dhopesh et al., 1997). The absence of correlation could be explained by mostly parallel processing of information by nigral and ventral tegmental dopaminergic fibers, respectively, with completely different projection targets, so that changes in one system will not alter the functioning of another. However, at the genetic and molecular level it is reasonable to expect similar molecules and pathways to be involved in both drug addiction and Parkinson's disease. Some examples are discussed below.

1. The dopamine transporter (DAT) is responsible for the removal of dopamine from the synaptic cleft after its release under normal conditions, and is the target both for selective dopaminergic neurotoxins (MPTP and 6-OHDA, used for simulation of parkinsonism-like states in animals), and for psychostimulant drugs such as cocaine or amphetamine. It is unknown whether the causes of neurotoxin-induced parkinsonian symptoms are the same in the etiology of Parkinson's disease in humans, but there is evidence that DAT is uniquely expressed on neurons that are primary targets of parkinsonian neurodegeneration. Transgenic mice with TH promoter-driven DAT over-expression show over 50% greater loss of dopaminergic neurons following MPTP treatment than wild-type control mice. These same mice display enhanced reward conferred by cocaine, as measured by conditioned place preference. These demonstrate the importance of DAT for both substance abuse vulnerability and dopaminergic neurodegeneration (Donovan et al., 1999). Chronic cocaine treatment and also 6-OHDA induced Parkinson's disease both significantly reduced the levels of DAT mRNA in the rat SN, pars compacta and VTA (Burchett and Bannon, 1997; Letchworth et al., 1999). The changes in the TH mRNA levels somehow mirror that of DAT and is increased in SN upon chronic cocaine treatment as well as in patients with either PD and AD (Vrana et al., 1993; Ito et al., 1999). Common adaptive processes involving the dopaminergic system may occur in different pathological conditions and also during aging. DAT and TH mRNA decrease in the SN with age, a fact probably relevant for the observed increased susceptibility to Parkinson's in elderly and also for the age-dependent sensitivity to drug actions, youngest subjects (rhesus monkeys) being less sensitive to cocaine effects on several brain functions than older ones (Paule et al., 1998).
2. As another example, the kappa opioid receptor (KOR) mRNA is down-regulated in the VTA and the NAcc by chronic ethanol and repetitive cocaine (Spangler et al., 1996; Rosin et al., 1999) while it is expressed at very low levels in the SN from Parkinson's patients and may play a role in the etiology of the disease (Yamada et al., 1997). On the other hand there is a loss of enkephalin- and dynorphin-like immunoreactivity in the SN (Waters et al., 1988) from Parkinson's patients, but subchronic administration of cocaine (20 mg/kg/day for 4 days) increases the striatonigral levels of dynorphin without altering the levels of Met5-enkephalin (Sivam, 1989).
3. An interesting correlation is also found between the involvement of glutamate transmission in parkinsonism and in cocaine action. Stimulation of NMDA receptors of the striatonigral projection may induce a slow neurodegeneration in Alzheimer's and Parkinson's disease due to a raise of the intracellular calcium concentration. Indeed nigrostriatal dopaminergic neurons containing calcium-binding proteins are less vulnerable to degeneration (Loopuijt and Schmidt, 1998), whereas neurotoxic effects of cocaine are strongest in the presence of anti-parvalbumin antibodies (Glezer et al., 1999). Also the development of behavioral sensitization towards cocaine parallels the elevation of extracellular glutamate in the VTA, probably by an increase in the capacity of neurons expressing D1 receptors to release glutamate after stimulation (Kalivas and Duffy, 1998), an effect probably mediated by neurotrophins. A significant increase in the expression of trkB is observed in the caudate-putamen ipsilateral to the 6-OHDA injection (Numan and Seroogy, 1997) while acute cocaine injection also results in a transient increase in NT-3 in the VTA and NT-3 contributes to the initiation of behavioral sensitization to cocaine by activating the Ras/MAP kinase signal transduction system (Pierce et al., 1999).
4. Furthermore both cocaine treatment (either acute or chronic) and the development of Parkinson's

disease alter the oxytocin content in hypothalamus (Purba et al., 1994; Sarnyai, 1998). Oxytocin-releasing cells of the paraventricular hypothalamic nucleus are also dopaminergic. Decreased oxytocin levels could provide a neural basis for some autonomic and endocrine disturbances in Parkinson's disease (Purba et al., 1994) but oxytocin also modulates different cocaine-induced behavioral phenomena (Sarnyai, 1998).

Together these data clearly demonstrate a correlation between the mechanisms involved in development of neurodegenerative diseases and in drug addiction. Nevertheless the precise function of γ-synuclein in cocaine action remains unexplained. From the protocol used in our present study, a role for γ-synuclein in long-lasting adaptations to the drug rather that in the acute response is suggested. Such a role is in agreement with functions proposed for γ-synuclein in the regulation of axonal growth and changes of axonal morphology, probably as a result to modifications of the integrity of the neurofilament network. Other plasticity related proteins, e.g. CART and EphA5, are also up-regulated following our protocol of drug treatment. CART has neurotrophic actions on dopaminergic neurons in vitro, increasing dopamine uptake, survival and neurite outgrowth (Louis, 1996). EphA5 is an axon guiding molecule involved in the establishment of mesostriatal dopaminergic projections during development (Yue et al., 1999). Therefore CART, EphA5 and γ-synuclein together could participate in local plasticity following cocaine treatment.

In conclusion, our study establishes an up-regulation of γ-synuclein in cocaine-treated brain which probably is associated with drug-induced plasticity changes in the dopaminergic system and suggests mechanisms common in the development of neurodegenerative diseases and drug addiction.

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Table 1. The distribution of α- and γ-synucleins mRNAs in rat brain. The relative intensity of expression is indicated (from +/- to +++ as the level of signal increases).

Brain region	**α-synuclein**	**γ-synuclein**
Cortex		
Layer II	+	+
Layer V	++	+/-
Other layers	-	+
Parietal, area 1	+/-	+
Insular	++	+
Piriform	+++	++
Other cortical areas	+	+
Claustrum	++	+
Dorsal endopiriform nucleus	+	+++
Ependyma	-	++
Subfornical organ	-	++
Olfactory system		
Anterior olfactory nucleus	+++	+
Nucleus of the lateral olfactory tract	+++	+
Tenia tecta	+++	+
Induseum griseum	++	+
Semilunar nucleus	-	++
Islands of Calleja	-	+++
Septum		
Nucleus of the horizontal limb of the diagonal band	+	+++
Nucleus of the vertical limb of the diagonal band	-	+
Lateral septal nucleus, dorsal part	+	+
Lateral septal nucleus, intermediate part	-	+
Lateral septal nucleus, ventral part	-	+
Basal ganglia		
Caudate putamen	+/-	+
Nucleus accumbens, shell–+	+/-	++
Nucleus accumbens, core	+/-	+
Ventral pallidum –	-	++
Olfactory tubercle	+/-	++
Claustrum ++	++	+
Hippocampus	+++	+
Thalamus		
Medial habenular nucleus	++	+
Paraventricular nucleus, posterior	++	++
Paraventricular nucleus, anterior	+++	++
Paratenial nucleus	-	++
Central medial nucleus	++	++
Rhomboid nucleus	++	++
Xiphoid nucleus	++	++
Intermediodorsal nucleus	++	++
Anteroventral nucleus	-	++
Ventral reuniens nucleus	-	++
Ventrolateral nucleus	-	++
Laterodorsal nucleus	-	++
Mediodorsal nucleus	-	++

Interanteromedial nucleus	-	++
Ventral posterolateral nucleus	-	++
Ventral posteromedial nucleus	-	++
Anteromedial nucleus	-	++
Reticular nucleus	-	++
Reuniens nucleus	-	++
Gelatinosus nucleus	-	++
Zona incerta	+	++
Subincertal nucleus	-	++
Hypothalamus		
Medial preoptic zone	-	++
Arcuate nucleus	-	+++
Supraoptic nucleus	-	+++
Paraventricular nucleus	–	+++
Periventricular nucleus	-	+++
Ventromedial nucleus	-	++
Dorsomedial nucleus	-	++
Magnocellular nucleus of the lateral hypothalamus	-	++
Suprachiasmatic nucleus	-	+++
Tuberomammillary nucleus	++	++
Medial preoptic nucleus	-	+++
Amygdala and extended amygdala		
Posterolateral cortical amygdaloid nucleus	++	+/-
Basomedial nucleus	+	+/-
Central nucleus	+++	+
Lateral nucleus	+	+
Basolateral nucleus	+	+
Bed nucleus of the stria terminalis	-	++
Amygdalohippocampal area	++	+/-
Amygdalohippocampal area, posteromedial part	+++	+/-
Amygdalopiriform transition area	+++	+/-
Midbrain		
Substantia nigra, pars compacta	+	++
Substantia nigra, pars lateralis	+/-	+/-
Substantia nigra, pars reticulata	-	-
Red nucleus, magnocellular part	+/-	+++
Ventral tegmental area	-	-
Edinger-Westfal nucleus	+/-	+++
Oculomotor nucleus	-	++
Interpeduncular nucleus	-	++

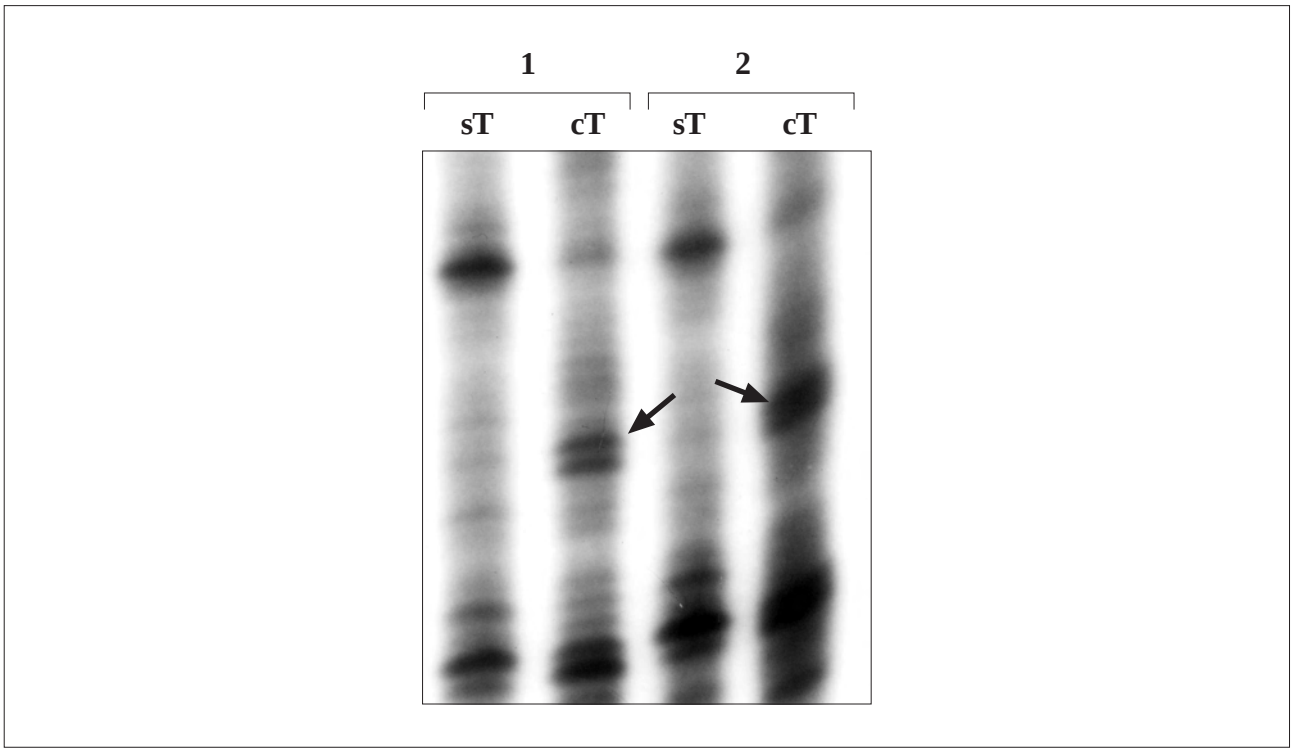

Figure 1. γ-synuclein over-expression in differential display. sT: saline tegmentum, cT: cocaine tegmentum; 1 and 2 correspond to two duplicate PCR reactions starting from independent reverse transcription reactions; γ-synuclein band is indicated by arrows (at approximately 120 bp).

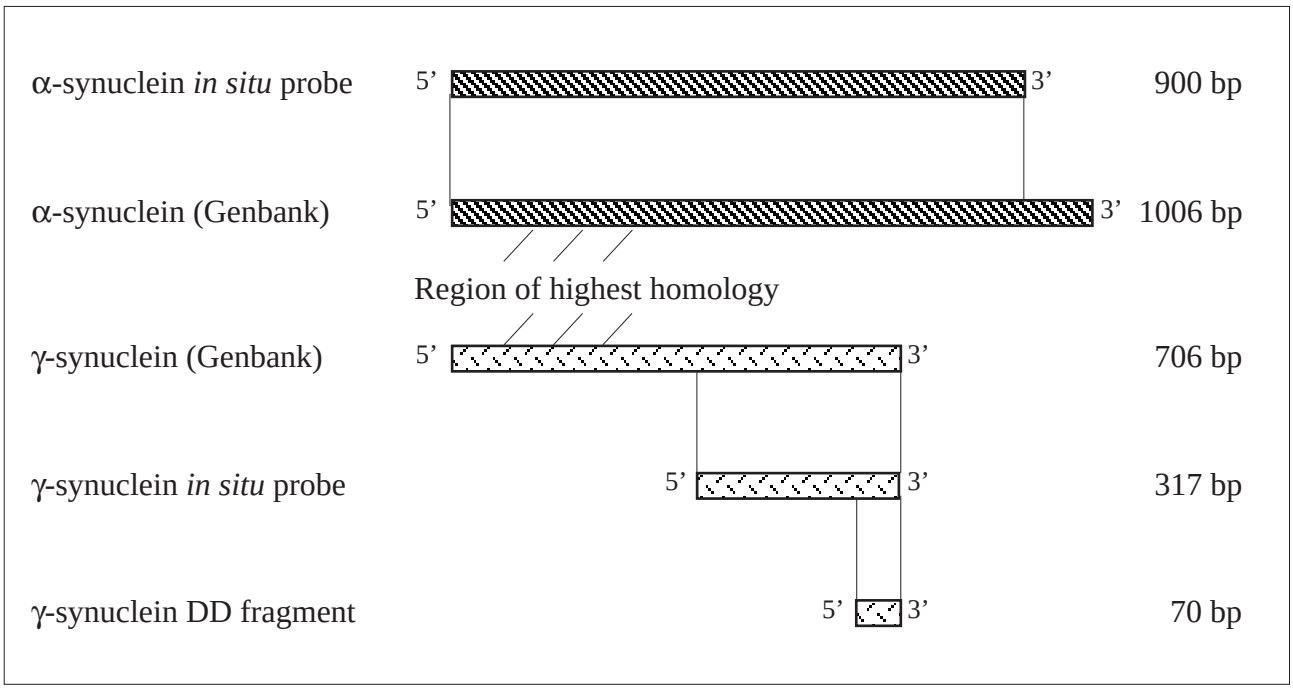

Figure 2. Alignment of synuclein tags used in the present study with the sequences from Genbank. Genbank mRNA sequences are given without poly(A) tail. The region of highest homology between two synucleins is indicated. γ-synuclein DD fragment: the sequence of tag found to be up-regulated in tegmentum upon cocaine in differential display experiment.

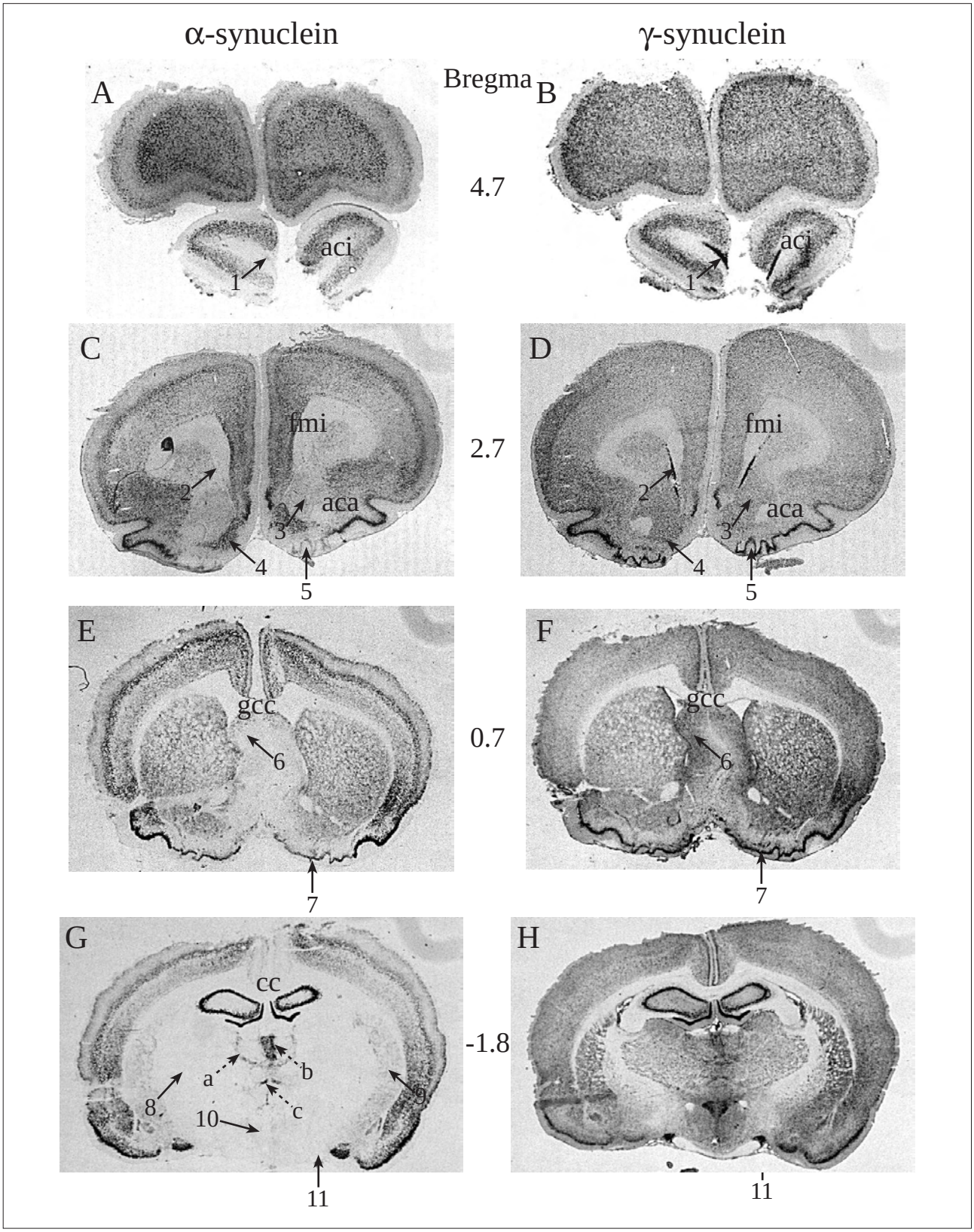

Figure 3. ***In situ*** **hybridization of synucleins mRNA distribution throughout the adult rat brain.** Photomicrographs are shown for coronal 25 μm sections hybridized with α- (A,C,E,G) or γ- (B,D,F,H) synucleins probes. To facilitate the localization of particular structures, Bregma stereotaxic coordinates are indicated. In addition, some key structures are labeled by abbreviations: aca: anterior commissure, anterior part; aci: anterior commissure, intrabulbar part; cc: corpus callosum; fmi: forceps minor of the corpus callosum; gcc: genu of the corpus callosum. Note the differential distribution of two mRNAs in the cortex and especially on the coronal section at Bregma –1.8. α-synuclein staining is predominantly present in the cortex, in the hippocampus and in few thalamic nuclei, labeled by dashed arrows and letters a-c. γ- synuclein staining is more widespread. Other differentially labeled regions are indicated by numbered arrows: 1: ependymal layer of the olfactory ventricle; 2: lateral ventricle; 3: nucleus accumbens; 4: anterior olfactory nucleus; 5: olfactory tubercle, pyramidal layer; 6: lateral septal nucleus; 7: olfactory tubercle; 8: ventral thalamic nuclei; 9: caudate putamen; 10: paraventricular hypothalamic nucleus; 11: supraoptic nucleus. a: central medial thalamic nucleus; b: paraventricular thalamic nucleus; c: rhomboid thalamic nucleus.

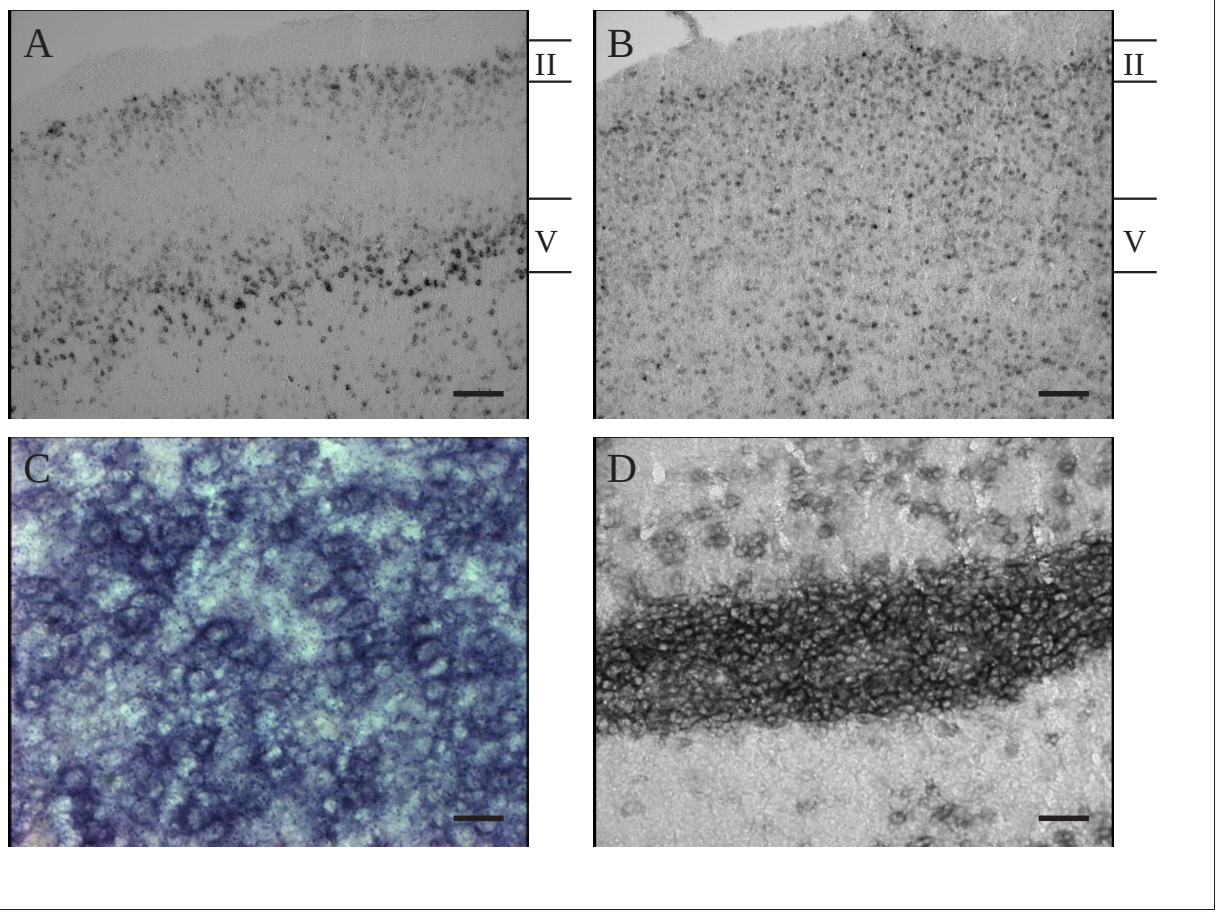

Figure 4. Expression of synucleins in the cortex, subfornical organ and ependyma. In the cerebral cortex, the expression of α-synuclein is confined primarily to cortical layers II and especially V (A), whereas γ-synuclein is present at lower intensities but distributed equally at all layers (B). Cortical layers are indicated by roman numbers. C: γ-synuclein staining in the subfornical organ. D: γ-synuclein in the ependymal cells lining the lateral ventricle. Scale bar: 20 μm (in A,B,E), 25 μm (in C) and 50 μm (in D).

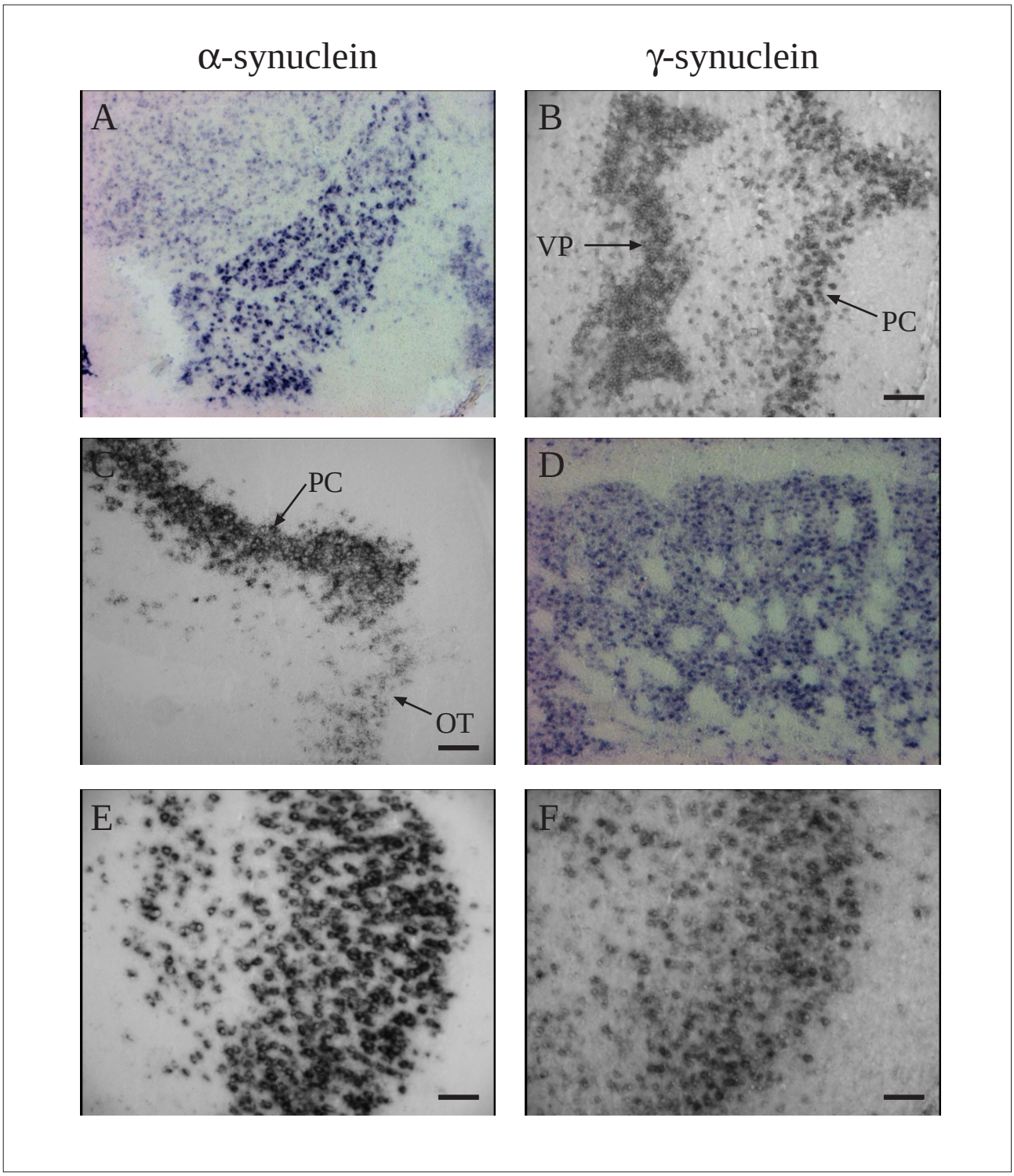

Figure 5. Expression of synucleins in the olfactory system and basal ganglia. α-synuclein staining is shown in panels A,C,E; γ-synuclein – in panels B,D,F. A: anterior olfactory nucleus; B: ventral pallidum (VP) and piriform cortex (PC); C: piriform cortex (PC) and olfactory tubercle (OT); D: caudate-putamen. Note the specific labeling by γ-synuclein of matrix compartment of the striatum. E,F: nucleus of the lateral olfactory tract. Scale bar 100 μm.

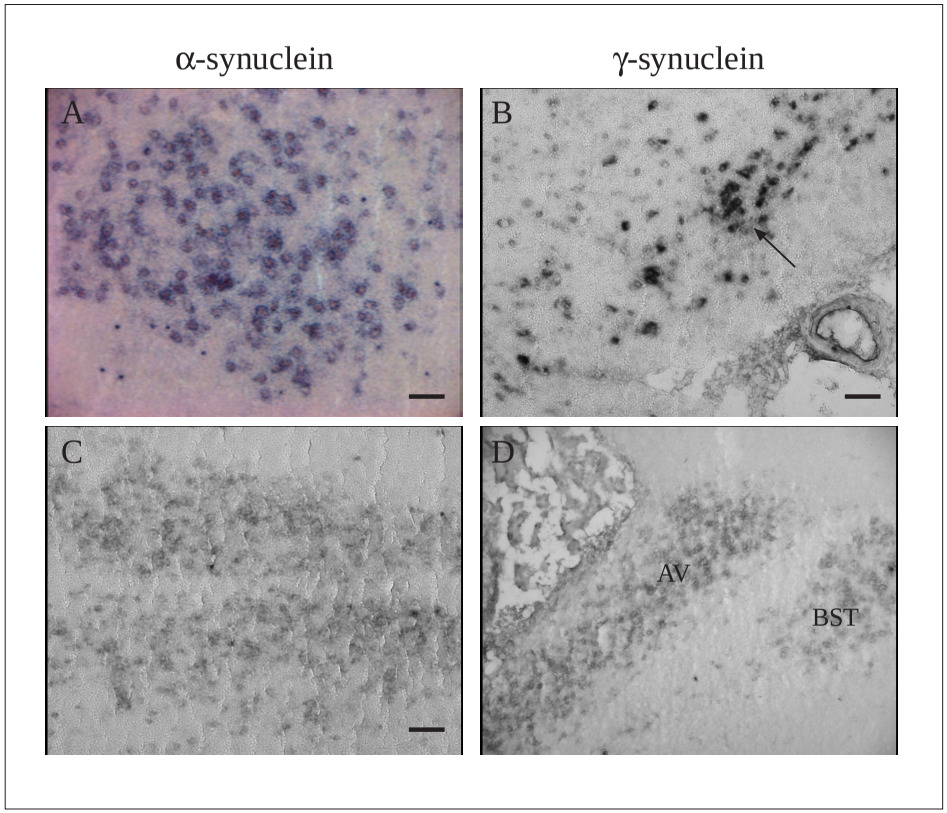

Figure 6. Expression of synucleins in the extended amygdala and thalamus. α-synuclein staining is shown in panels A,C; γ-synuclein – in panels B,D. A: basolateral amygdaloid nucleus; B: nucleus of the horizontal limb of the diagonal band, the group of highly positive for γ-synuclein cells is indicated by arrow. C: paraventricular thalamic nucleus; D: bed nucleus of the stria terminalis (BST) and the anteroventral thalamic nucleus (AV). Scale bar: 100 μm.

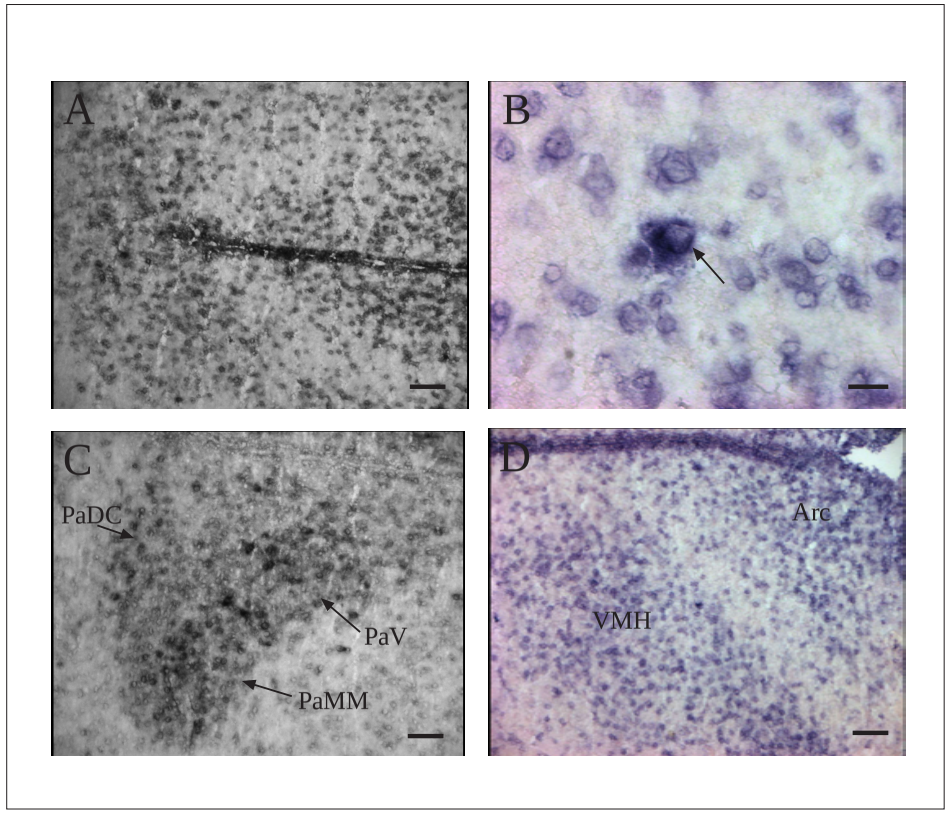

Figure 7. Expression of γ-synuclein in the hypothalamus. A: medial preoptic zone; B: staining in the lateral hypothalamic area, the large cell, highly positive for γ-synuclein, is indicated by arrow. C: paraventricular hypothalamic nucleus. Note the extensive γ-synuclein signal in the oxytocin-secreting cells (PaMM) and in the subregions regulating the autonomic nervous system (PaDC and PaV). PaDC: dorsal medial cap, PaMM: medial magnocellular division; PaV: ventral group. D: ventromedial (VMH) and arcuate (Arc) hypothalamic nuclei. Scale bar: 100 μm (in A, C, D), 25 μm (in B)

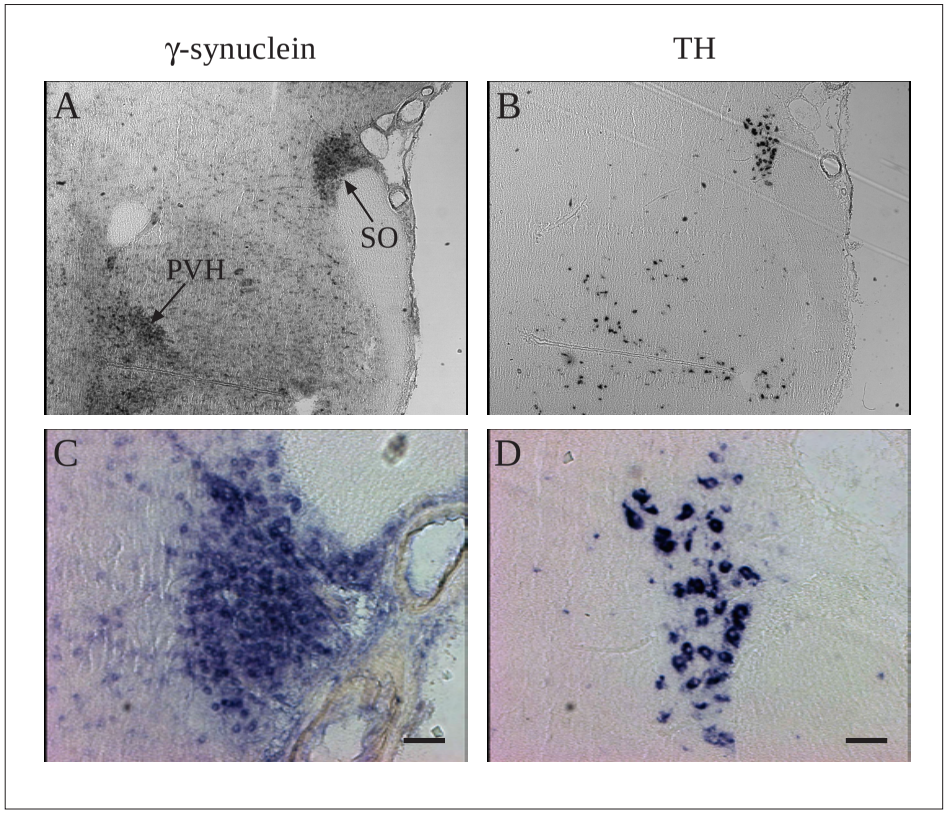

Figure 8. Expression of γ-synuclein and TH in the paraventricular (PVH) and supraoptic (SO) hypothalamic nuclei. γ-synuclein staining is shown in panels A,C; TH – in panels B,D. A,B: two nuclei at low magnification; C,D: supraoptic nucleus at higher magnification. Scale bar: 100 μm.

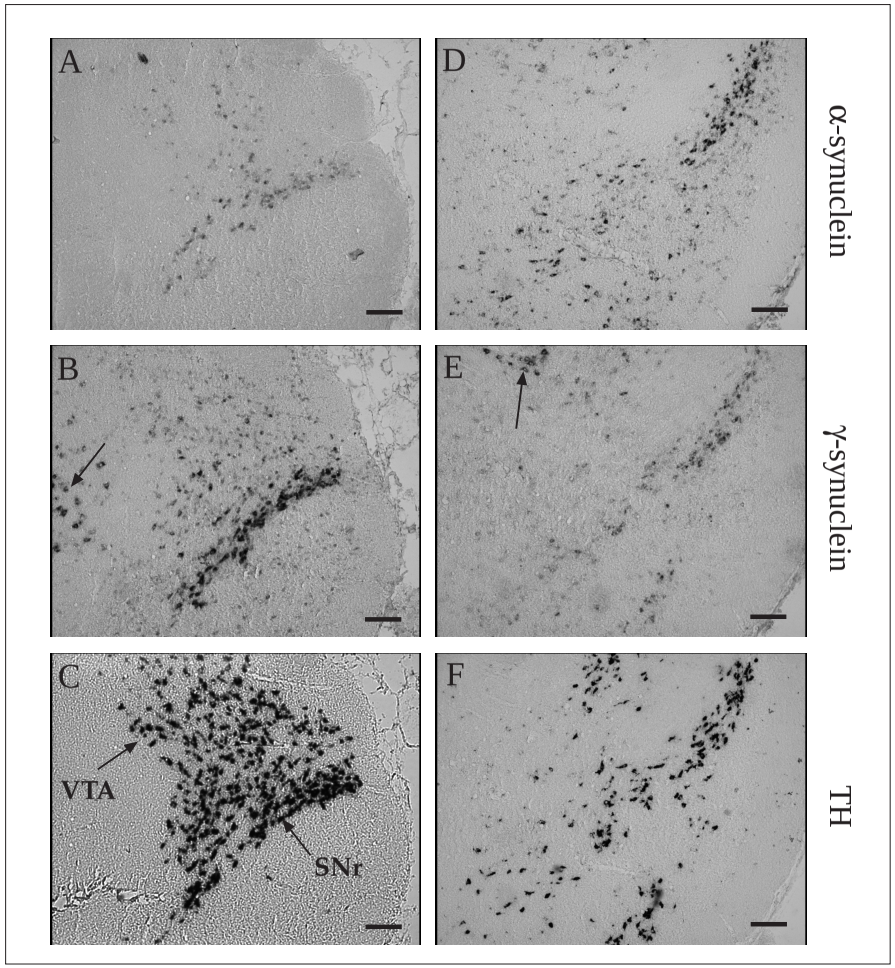

Figure 9. Expression of synucleins in midbrain DAergic nuclei. A-C and D-F are the two examples of *in situ* hybridization experiments performed on three adjacent sections with α- (A,D), γ-synuclein (B,E) and TH (C,F) probes respectively. α-synuclein is more restricted and correlates mostly with TH expression (A,C,D,F). γ-synuclein is less TH-cell specific and is present also outside DAergic nuclei (arrows in B and E). Note that both synucleins are present predominantly in the substantia nigra (SN) and practically absent in the ventral tegmental area (VTA) (A-C). Scale bar: 200 μm.

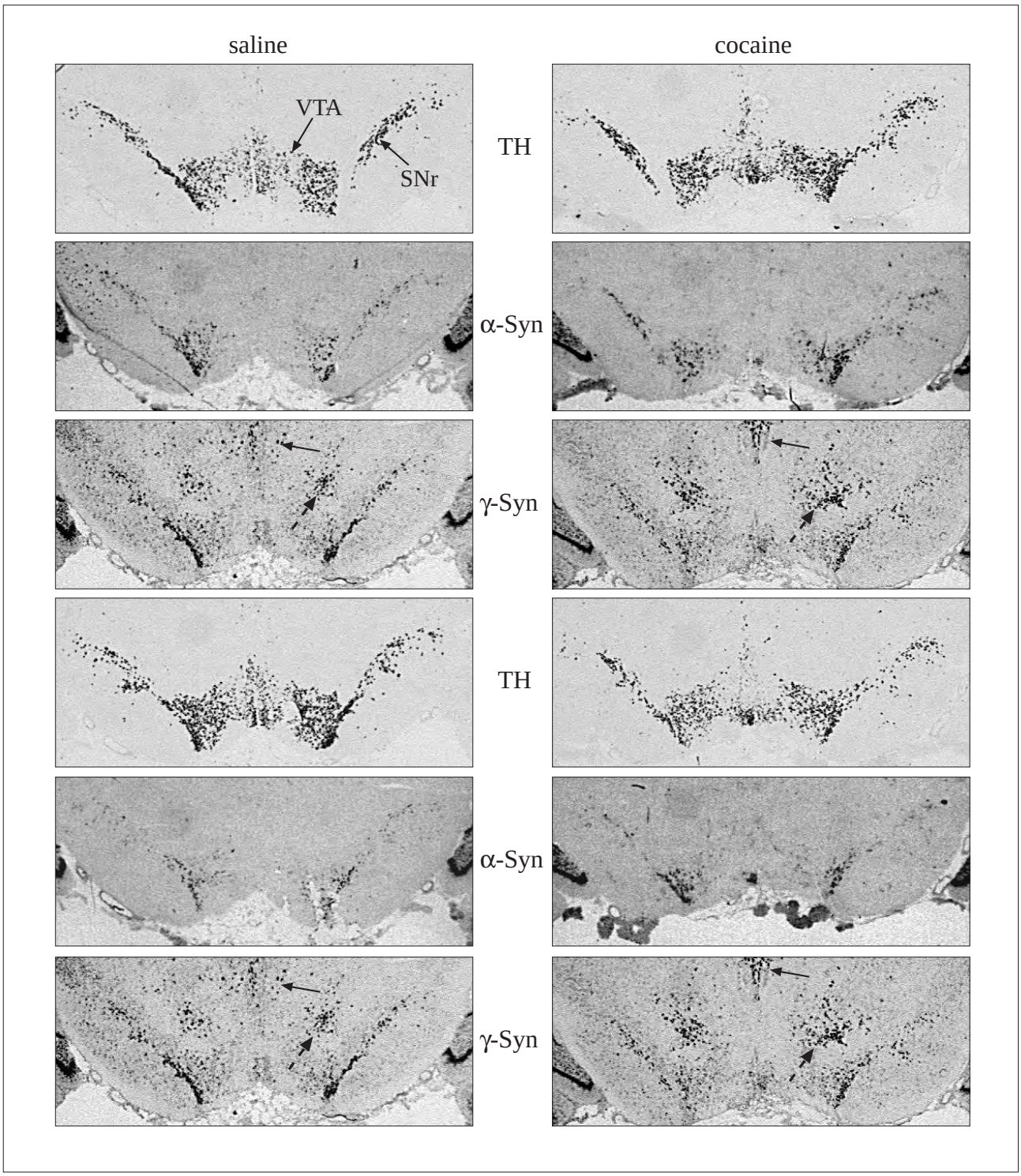

Figure 10. Expression of synucleins in saline- and cocaine-treated brains. Probes for TH, α-, and γ-synucleins were hybridized to six subsequent coronal sections of brains from saline- or cocaine-treated animals. The Edinger-Westfal nucleus is indicated by arrows on γ-synuclein-stained sections. Note the lower and more scattered expression in saline-treated brain. The red nucleus is indicated by dotted arrows on γ-synuclein-stained sections. Note the higher signal in cocaine-treated brain. SN: substantia nigra; VTA: ventral tegmental area.

Curriculum Vitae

Personal

Name	Maria S. BRENZ VERCA-KOUZNETSOVA
Date and place of birth	August 20, 1970, Moscow
Marital status	Married, one child
Languages	Russian (native), English (fluent), French (fluent), Italian (passive)
Address	Institut de Biochimie, Université de Fribourg Rue du Musée 5, 1700 Fribourg, Switzerland Tel. (++41)-026-300-8634 Fax (++41)-026-300-9735 E-mail: Maria.Kouznetsova@unifr.ch

Education:

Social education	1978-1988: State Secondary School in Moscow with intensive study of English
High School	1988-1994: Moscow State University, College of Biology, Human and Animals Physiology Department

Research experience

1992-1993, prediploma work, Moscow University, Human and Animals Physiology Department, supervisor: Dr. A.Chepurnov, project: "Behaviour of rats in the labyrinths of different types and the influence of medical treatment on their ability of studying and memory". Methods used: animal handling, including behaviour tests and experimental induction of different pathologies.

1993-1994, diploma work, Moscow Cardiological Centre, supervisor: Dr. N.Golovanova, project: "Study of concentration of antibodies against gangliosides and serotonin in the blood of patients with cardio-vascular and endocrine diseases". Methods used: basic methods of lipids purification and characterisation including several chromatographic and electrophoretic techniques, ultracentrifugation, and also immunoassays (ELISA). Diploma work was attested as perfect.

September 1995, start of PhD Thesis at the Institute of Biochemistry of the University of Fribourg, supervisor: Prof. J.-L.Dreyer. Project: characterisation of genes expressed upon cocaine treatment in the rat mesolimbic dopaminergic system. The techniques used: differential display, in *situ* hybridisation, quantitative RT-PCR, immunohistochemistry and other methods of neuroscience and molecular biology.

Membership to societies and special programs

1996-present	member, Swiss Society for Cellular and Molecular Biology (http://www.sanw.unibe.ch/exthp/zmg/) (member of USGEB, the Swiss Union of Societies of Experimental Biology)
1997-present	member, Swiss Society for Neuroscience (SSN) (http://www.medecine.unige.ch/ssn/)
	1997-present participation in the postgraduate program for Neuroscience of the Universities of Fribourg and Bern (BENEFRI Neuroscience) (http://pylwww.unibe.ch/benefri/)
	1998-present member, Société Fribourgeoise de Chimie (SFC)
1999-present	member, Société des Neurosciences (http://www.neurosciences.asso.fr/)
1999-present	member, Society for Neuroscience (SFN) (http://www.sfn.org/)

Practical courses

Oct., 1995	AN INTRODUCTION TO PCR AND HYBRIDISATION TECHNIQUES; Organized by Prof. I.J.Bruce and Dr. C.D.Hurst, Bern, Switzerland
March, 1996	EXPRESSION OF A PROTEIN OF MEDICAL INTEREST IN HETEROLOGOUS SYSTEMS; IIIeme Cycle Romand en Sciences Biologiques (http://www.3eme-cycle.ch/), Lausanne, Switzerland
Spring, 1997	PROFESSIONAL COMMUNICATION SKILLS IN ENGLISH FOR ACADEMIC RESEARCHERS; Organized by Anthony Clark, English Language Unit, University of Fribourg Language Centre, Switzerland
Sept., 1997	SEQUENCE ANALYSIS; EMBNet / IIIeme Cycle Romand en Sciences Biologiques, Lausanne, Switzerland
May, 1998	*IN SITU* HYBRIDISATION; SKMB (Swiss Kommission for Molecular Biology), Bern, Switzerland

Sept., 1999	CONSTRUCTION AND HANDLING OF RECOMBINANT ADENOVIRUSES; IIIeme Cycle Romand en Sciences Biologiques, Fribourg, Switzerland
March-June 2000	Practical course of neuroanatomy: HUMAN CENTRAL NERVOUS SYSTEM, Institute of Anatomy, University of Fribourg, Switzerland

Attended meetings and lecture cycles

Oct., 1996	Meeting on DIFFERENTIAL DISPLAY AND RELATED TECHNIQUES FOR GENE DISCOVERY, Cold Spring Harbor Laboratory, USA. Poster and oral presentation: "A new strategy for DD RT-PCR allows very fast identification and cloning of differentially expressed genes"
March, 1997	Annual meeting of the Swiss Union of Societies of Experimental Biology (USGEB), Lausanne, Switzerland. Poster: "Identification of genes involved in oxidative stress response in neuronal cells by an improved differential display method"
Sept., 1997	Lecture cycle on BIOLOGICAL DEFENSE MECHANISMS; IIIeme Cycle Romand en Sciences Biologiques, Villars-sur-Ollon, Switzerland. Poster: "Identification of genes involved in oxidative stress response in neuronal cells by an improved differential display method"
Jan., 1998	Joint Meeting of the Swiss Society for Neuroscience (SSN) and Swiss Society of Biological Psychiatry (SSBP), Lausanne, Switzerland
March, 1998	BENEFRI block course in neuroscience 1998, Fribourg, Switzerland
March, 1998	Annual meeting of the Swiss Union of Societies of Experimental Biology (USGEB), Genève, Switzerland. Poster: "Cloning of rat brain collapsin genes"
June-Jul., 1998	1998 Forum of European Neuroscience, Berlin, Germany. Poster: "DDRT-PCR analysis of differential gene expression in the rat mesolimbic dopaminergic system after cocaine-withdrawal"
Jul., 1998	DOPAMINE 98 (Satellite symposium of the IUPHAR congress in Munich), Strasbourg, France. Poster: "DDRT-PCR analysis of differential gene expression in the rat mesolimbic dopaminergic system after cocaine-withdrawal"
Sept., 1998	Lecture cycle on PATTERN FORMATION IN PLANTS AND ANIMALS; IIIeme Cycle Romand en Sciences Biologiques, Villars-sur-Ollon, Switzerland
Jan., 1999	Swiss Society for Neuroscience Annual Meeting (4th Joint Meeting of the SSN-SSBP), Zurich, Switzerland. Poster: "Differential gene expression in the mesolimbic dopaminergic pathway after cocaine withdrawal"
March, 1999	BENEFRI block course in neuroscience 1999 on MOTOR CONTROL: PHYSIOLOGY, MOLECULAR BIOLOGY AND CLINICS, Bern, Switzerland
May, 1999	4ème Colloque de la Société des Neurosciences, Marseille, France
Sept., 1999	Symposium on RECOMBINANT ADENO 99: SOMEWHERE BETWEEN THE LAB AND THE CLINICS; IIIeme Cycle Romand en Sciences Biologiques, Fribourg, Switzerland
Sept., 1999	Lecture cycle on CHROMOSOMES: DOMAINS AND DYNAMICS; IIIeme Cycle Romand en Sciences Biologiques, Villars-sur-Ollon, Switzerland
Oct., 1999	Society for Neuroscience 29th Annual Meeting, Miami Beach, Fla
Dec., 1999	Workshop on the NEURAL MECHANISMS OF ADDICTION, Madrid, Spain. Poster: “CD81 expression after acute cocaine treatment in the adult rat brain”
March 2000	BENEFRI block course in neuroscience 2000 on THE HYPOTHALAMUS: INTEGRATED BEHAVIORAL, AUTONOMIC, AND ENDOCRINE CONTROL. Organised by Clifford B.Saper and Marco R.Celio, Fribourg
May 2000	International workshop on BASAL GANGLIA AND THALAMUS IN HEALTH AND MOVEMENT DISORDERS, Moscow, Russia (http://www.anatomy.uiowa.edu/bgthmd/workshop3.html). Poster: “Expression of synucleins, a family of parkinson-related proteins, in normal and cocaine-treated adult rat brain”
June 2000	Federation of European Neuroscience Societies (FENS) 2000, Brighton, UK (http://www.fens2000.org/). Poster: “Synucleins expression in normal and cocaine-treated adult rat brain”
Jul., 2000	Symposium on ESSENTIAL MECHANISMS IN BRAIN FUNCTION; IIIeme Cycle Romand en Sciences Biologiques, Lausanne, Switzerland

Teaching experience

1996-1999	Practicals in biochemistry, 2nd year medical students, 1 afternoon / 2 weeks.
March 1998	Advanced practical course in biochemistry on PURIFICATION AND ANALYSIS OF cDNA TAGS FOUND BY DIFFERENTIAL DISPLAY, 3rd year biochemistry students.

List of publications:

1) Brenz Verca-Kouznetsova M.S., Brenz Verca S., Rusconi S., Dreyer J.L. “Modification of Primer Design Facilitates the Use of Differential Display” BioTechniques 24:374-380 (March 1998)
2) Brenz Verca-Kouznetsova M.S., Brenz Verca S., Rusconi S., Dreyer J.L. “Modification of Primer Design Facilitates the Use of Differential Display” in: Expression Genetics: Differential Display, Edited by Arthur Pardee and Michael McClelland, 1999, Biotechniques Books, Natick, MA
3) Brenz Verca M.S., Widmer D.A.J., Wagner G.C. and Dreyer J.-L. “Cocaine-induced expression of the tetraspanin CD81 and its relation to hypothalamic function” (accepted for publication in Molecular and Cellular Neuroscience)
4) Michna L., Brenz Verca M.S., Widmer D.A.J., Chen S., Lee J., Rogove J., Zhou R., Tsitsikov E., Miescher G.C., Dreyer J.-L. and Wagner G.C. “Induction of CD81 by Cocaine in Rats and Altered Sensitivity of CD81-Deficient Mice to Neurobehavioral Effects of Cocaine” (submitted)
5) Brenz Verca M.S., Widmer D.A.J., Wagner G.C. and Dreyer J.-L. “Cocaine-regulated expression of γ-synuclein in the adult rat brain after acute drug withdrawal” (in preparation)

Déclaration

Fribourg, Décembre 2000

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Aux personnes concernées

Sujet: Thèse présentée à l'Université de Fribourg (Suisse) pour l'obtention du grade de *Doctor rerum naturalium*

Messieurs,

Par la présente, je certifie que j'ai rédigé ma thèse moi-même et sur la base d'un travail personnel sans aide illicite.

Maria Brenz Verca-Kouznetsova