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# POSTNATAL DEVELOPMENT OF GLUTAMATERGIC RECEPTOR-MEDIATED EXCITATORY POSTSYNAPTIC CURRENTS AND THEIR MODULATIONS BY ACH AND DOPAMINE IN NUCLEUS ACCUMBENS

Par
Liming Zhang

Département de Physiologie
Faculté de Médecine

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Présentée par

Liming Zhang

a été évaluée par un jury composé des personnes suivantes

Dr. Jacques de Champlain
Dr. Jean-Claude Lacaille
Dr. Ariel Agmon
Dr. Richard A Warren

, représentant de la F.E.S.

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## SOMMAIRE

Ce travail décrit le développement postnatal des courants postsynaptiques excitateurs (CPSEs) dans les neurones épineux moyens (MS) du noyau accumbens (nAcb) du rat in vitro ainsi que les effets neuromodulateurs de l'acétylcholine (ACh) et de la dopamine (DA) en utilisant la technique *whole-cell patch-clamp*.

Les CPSEs, évoqués par une stimulation électrique, ont été enregistrés dans plus de 500 neurones du nAcb pendant le développement postnatal à partir du jour de la naissance (jour postnatal 0; P0) jusqu'à P71. Un CPSE a été identifié dans tous les neurones enregistrés et à tous les âges, démontrant que des synapses excitatrices fonctionnelles étaient déjà présentes dans le nAcb le jour de la naissance. Dans la majorité de neurones (80%), les CPSEs avaient deux composantes distinctes. La première atteignait un maximum entre 4 et 21ms après le début de stimulus, avait une relation $I_R$-$V_M$ linéaire et était sensible au CNQX. La seconde composante pouvait être mesurée entre 20 et 138 ms après le début de stimulus, avait une relation $I_R$-$V_M$ en v avec un maximum autour de –40 mV et était sensible à l'APV. Ces caractéristiques montrent que la composante précoce des CPSEs étaient médiée par des récepteurs de type AMPA/KA tandis que la deuxième était médiée par des récepteurs de type NMDA.

Pendant les premiers jours suivant la naissance, l'amplitude des CPSEs était relativement petite. Par la suite, les CPSEs ont augmenté progressivement jusqu'à la fin de la deuxième semaine postnatale. À partir de ce moment, l'amplitude de la composante précoce s'est stabilisée jusqu'à l'âge adulte alors que celle de la composante tardive a commencé à diminuer pour devenir virtuellement nulle dans les préparations provenant d'animaux âgés de plus de 3 semaines. Le rapport entre l'amplitude de la composante

tardive et celle de la composante précoce a augmenté graduellement durant les deux premières semaines et a par la suite diminué de façon marquée. Ces résultats suggèrent que l'expression de CPSEs médiés par les récepteurs NMDA est prédominante durant la deuxième semaine du développement postnatal dans le nAcb.

Nous avons trouvé que l'ACh produisait deux effets médiés par différents types de récepteurs sur les CPSEs. L'ACh diminuait les CPSEs en agissant sur des récepteurs muscariniques tandis qu'elle augmentait les CPSEs en agissant sur des récepteurs nicotiniques. Cependant, l'effet excitateur produit par l'activation des récepteurs nicotiniques était généralement masqué par les effets inhibiteurs muscariniques en absence d'un antagoniste de ces derniers. Donc, l'activation des interneurones cholinergiques dans le nAcb pourrait produire une excitation nicotinique rapide et une inhibition muscarinique plus lente.

La DA, par une action sur les récepteurs de la famille D1, produisait une diminution importante du rapport entre les CPSEs médiés par des récepteurs NMDA et ceux médiés par les récepteurs AMPA/KA en inhibant davantage les CPSEs médiés par les récepteurs NMDA. En effet, l'inhibition produite par la DA sur les CPSEs NMDA était comparable à celle produite par l'APV, un antagoniste spécifique des récepteurs NMDA. Les effets de la DA sur les CPSEs ne semble pas impliquer la protéine kinase A ni la protéine kinase C, parce que cette action était résistante aux inhibiteurs de protéines kinases H89 et Ro-32-0432.

Les agonistes cholinergiques et dopaminergiques ont changé le rapport des réponses à des stimuli pairés (*paired pulse ratio*) sans toutefois modifié la conductance membranaire ni la réponse des neurones au glutamate en présence de TTX, suggérant que

leurs effets sur les CPSEs étaient principalement médiés par des mécanismes présynaptiques. Cependant, les effets postsynaptiques de l'ACh et et de la DA ont pu être masqués par la présence de QX-314 dans la pipette d'enregistrement car cette substance bloque certains canaux ioniques $K^+$ et $Na^+$ qui auraient pu être modulés par l'ACh et la DA.

En résumé, cette recherche démontre que les réponses synaptiques médiées par les récepteurs NMDA atteignent leur maximum pendant la seconde semaine du développement postnatal et pourraient jouer un rôle important dans les processus développementaux dépendant de l'activité. La modulation des CPSEs NMDA et AMPA/KA pendant cette période par la DA et l'ACh suggèrent que ces substances pourraient jouer un rôle déterminant aussi pendant cette période.

**Mots-clés** : CPSE, développement postnatal, Acétylcholine, dopamine, récepteur AMPA/KA, récepteur NMDA, noyau accumbens, courant postsynaptique excitateur

## SUMMARY

This work describes the postnatal development of excitatory postsynaptic currents (EPSCs) of medium spiny neurons (MS) in nucleus accumbens (nAcb) slices of rat as well as the modulations of two classic neurotransmitters dopamine (DA) and acetylcholine (ACh) on EPSCs using whole-cell patch-clamp technique.

EPSCs were evoked by local electrical stimulation in 509 nAcb neurons during postnatal development from the day of birth (postnatal day 0; P0) to P71. An EPSC was found in all recorded neurons of all ages, showing that functional excitatory synapses were already present in the nAcb on the day of birth. In majority of neurons (80%) the EPSCs had two distinct components: an early component with a peak between 4 and 21 ms after stimulus onset, linear $I_R$-$V_m$ and sensitive to CNQX and a late one that was found from 20 to 138 ms after stimulus onset, had a V-shape $I_R$-$V_m$ relationship with a peak around –40 mV and sensitive to APV. These characteristics demonstrate the early and late components of the EPSC were mediated by AMPA and NMDA receptors respectively. During the first few days after birth, the amplitudes of both early and late components of the EPSCs were relatively small and then started to increase until the end of the second postnatal week. Whereas the early component of the EPSC appeared to stabilize from that point on, the late component began to decrease in samples from animals aging more than 3-week-old. In addition, the ratio between the amplitudes of late and early components followed a developmental pattern gradually increased during the first two postnatal weeks followed by a decrease dramatically. Together, these results show that there is a dominant expression of NMDA receptor-mediated EPSC during the postnatal development in nAcb.

Two remarkable differential actions of ACh were found on the EPSCs by two types of ACh receptors. ACh depressed EPSCs through muscarinic M1 receptors, whereas it enhanced EPSCs through nicotinic receptors, suggesting that nAcb cholinergic interneurons may produce a fast nicotinic excitation and slow muscarinic inhibition. However, nicotinic receptor-mediated effects were usually masked by muscarinic receptor-mediated actions in our experimental condition *in vitro*. Moreover, we also found that the inhibitory effects of ACh on NMDA receptor- but not on AMPA receptor-mediated EPSCs significantly increased during the first two postnatal weeks.

DA, through an action on $D_1$-like receptor, distinctly decreased the ratio of NMDA receptor- to AMPA receptor-mediated EPSCs, mimicked the effect of APV on EPSCs and abolished almost completely NMDA receptor-mediated EPSCs with minimal effect on AMPA receptor-mediated EPSCs. The DA-induced depression of EPSCs did not involve either protein kinase A or protein kinase C, because this action was resistant to the protein kinase inhibitors H89 and Ro-32-0432.

Both cholinergic and dopaminergic agonists altered the ratio of paired-pulse stimulation-evoked EPSCs, but did not change input conductance of membrane of MS neurons. Also both of transmitters had no effect on glutamate injection-evoked EPSCs in the presence of TTX and QX-314, suggesting their modulation on EPSCs is mainly presynaptic in the postnatal development of nAcb. However, in accordance with the effect of QX-314 on membrane potentials during the modulation of ACh, and distinct change in the ratio of NMDAR/AMPAR-EPSCs during the modulation of DA, it is suggested that there are postsynaptic effects of ACh and DA either in the nAcb during the postnatal development. This work shows that NMDA receptor-mediated synaptic

responses are dominant during postnatal development and that DA and ACh effectively modulate NMDA receptor-mediated currents, indicating that NMDA receptor-mediated synaptic events may play important roles in postnatal development. The modulations of NMDA receptor- and AMPA receptor-mediated EPSCs by DA and ACh may be important for activity-dependent developmental processes or other plasticity in the nAcb. In addition, quite significant modulation of NMDA receptor-mediated EPSCs by ACh and DA during postnatal development could be important for the study in the etiology of schizophrenia.

**Key words**: Nucleus accumbens, Excitatory postsynaptic currents, Postnatal development, Cholinergic and dopaminergic modulation, NMDA receptor, AMPA receptor, Pre and postsynaptic modulations.

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## LIST OF ABBREVIATIONS

| | | |
|---|---|---|
| ACh | : | Acetylcholine |
| AChR | : | Acetylcholine receptor |
| ACSF | : | Artificial cerebrospinal fluid |
| AMP | : | Adenosine monophosphate |
| AMPA | : | α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate |
| AMPA/KA | : | AMPA/kainate |
| AMPAR- | : | AMPA receptor-mediated |
| APV | : | D(-)2-amino-5-phosphonopentanoic acid |
| ATP | : | Adenosine triphosphate |
| BAPTA | : | Bis(2-aminophenoxy)ethane-N,N,N', N'-tetraacetate |
| BMI | : | Bicuculline methiodide |
| $Ca^{2+}$ | : | Calcium |
| cAMP | : | Cyclic adenosine monophosphate |
| CCh | : | Carbachol |
| ChAT | : | Choline acetyl transferase |
| $Cl^-$ | : | Chloride |
| 7Cl Kyn | : | 7-chlorokynurenic |
| CNQX | : | 6-cyano-7-nitroquinoxaline-2,3-dione |
| CNS | : | Central nervous system |
| CTZ | : | Cyclothiazide |
| DA | : | Dopamine |
| DAG | : | Diacylglycerol |
| DHβE | : | Dihydro-β-erythoidine |
| DMPP | : | 1,1-dimethyl-4-phenylpiperazinium |
| DMSO | : | Dimethlysulfoxide |
| DOPAC | : | 3,4-dihydroxyphenylacetic acid |
| EPSCs | : | Excitatory postsynaptic currents |
| EPSP | : | Excitatory postsynaptic potential |
| FS | : | Fast spiking |
| GABA | : | γ-aminobutyric acid |
| GluRs | : | Glutamate receptors |
| G-protein | : | Guanine nucleotide binding protein |
| GTP | : | Guanine triphosphate |
| HVA | : | homovanillic acid |
| $IP_3$ | : | Inositol 1,4,5 triphosphate |
| IPSC | : | Inhibitory postsynaptic current |
| IPSP | : | Inhibitory postsynaptic potential |
| $K^+$ | : | Potassium |
| KA | : | Kainate |
| LA | : | Large aspiny |
| LTS | : | Low threshold spike |
| mAChR | : | Muscarinic ACh receptor |

| | | |
|---|---|---|
| McN-A-343 | : | 4-[[[(3-chlorophenyl)amino]carbonyl]oxy]-N,N,N-trimethyl-2-butyn-1-aminium chloride |
| MLA | : | Methyllycaconitine citrate |
| MS | : | Medium spiny |
| $Na^+$ | : | Sodium |
| nAcb | : | Nucleus accumbens |
| nAChR | : | Nicotinic ACh receptor |
| NMDA | : | N-methyl-D-aspartate |
| NMDAR- | : | NMDA receptor-mediated |
| NO | : | Nitric oxide |
| PFC | : | Prefrontal cortex |
| PKA | : | Protein kinase A |
| PKC | : | Protein kinase C |
| PLC | : | Phospholipase C |
| PP1 | : | Phosphatase 1 |
| RMP | : | Resting membrane potential |
| TTX | : | Tetrodotoxin |
| VTA | : | Ventral tegmental area |

To my parents

To my wife and daughter

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# CHAPTER I

# INTRODUCTION

## 1. Overview

The nucleus accumbens (nAcb) is an important point of convergence for different afferents originating in limbic structures (Lopes da Silva, 1984; Pennartz and Kitai, 1991; Pennartz et al., 1994; O'Donnell and Grace, 1995; Finch, 1996). Several of these pathways are thought to be glutamatergic and to provide an excitatory drive by activating NMDA and/or AMPA/KA receptors necessary to trigger firing activity in nAcb neurons (Kombian and Malenka, 1994). In addition, the nAcb receives a dense dopaminergic input from the ventral tegmental area (VTA), and this system has been implicated in drug addiction and other neuropsychiatric disorders (Ungerstedt, 1971; Schilstrom et al., 1998a,b). The nAcb also contains a small population of cholinergic interneurons, which play an important role in modulating glutamatergic transmission (Sugita, et al., 1991; Hersch, et al., 1994; Zhang and Warren, 2002; de Rover et al., 2002).

The nAcb has been proposed to serve as an interface between the limbic system and the extrapyramidal motor system (Mogenson et al., 1980; Mogenson and Yim, 1981; Powell and Leman, 1976; Yang and Mogenson, 1984; Yim and Mogenson, 1982). Studies have provided evidence for the involvement of the nAcb in a number of functions including motivation (Mogenson et al., 1980; Robbins and Everitt, 1996; Swerdlow and Koob, 1987), attention (Solomon and Staton, 1982; van den Bos et al., 1991), and reward (Apicella et al., 1991; Colle and Wise, 1988; Robbins and Everitt, 1996; Fantin and Bottecchia, 1984; Olds, 1990). Recent studies have shown its involvement in learning and plasticity (Parkinson et al., 2000). Moreover, the nAcb may be involved in mediating some of the therapeutic actions of antipsychotic drugs that inactivate the mesencephalic

dopaminergic cells that project to this region when administered chronically (Chiodo and Bunney, 1983; White and Wang, 1983). Chaotic neurotransmissions in the nAcb could be a critical determinant in some neuropsychiatric disorders, including schizophrenia, Tourett's syndrome and drug addiction (Koob and Nestler, 1997; Wise, 1998). Furthermore, selective loss of cholinergic interneurons in the nAcb has also been observed in schizophrenia (Holt et al., 1999) and Alzheimer's disease (Lehéricy et al., 1989). Additionally, an alteration of dopaminergic transmission is thought to play a key role in psychiatric disorders (Sokoloff et al., 1992), such as, dopaminergic hyperfunction has been implicated in schizophrenia (Gray et al., 1995; Joyce, 1993; Joyce and Meador-Woodruff, 1997). Interest in understanding cholinergic and dopaminergic mechanisms in controlling or regulating motor and psychological function in mammals has been growing since acetylcholine (ACh) and dopamine (DA) were postulated to play a role in the pathophysiology described above.

Ionotropic glutamate receptors-mediated events including excitatory postsynaptic currents (EPSC) play a crucial role in synaptogenesis and formation of neuronal circuitry, as well as in synaptic plasticity including long-term potentiation (LTP) and long-term depression (LTD). However, excessive activation of glutamate receptors might induce excitotoxic neuronal cell death, which is thought to contribute to neurodegeneration (Choi, 1992; Lipton and Rosenberg, 1994). Defects in glutamatergic transmission in the nAcb are thought to be involved in the pathophysiology of schizophrenia (Carlsson and Clarlsson, 1990a, b; Olney and Farber, 1995; Meador-Woodruff and Healy, 2000). However, during postnatal development, the characteristics of EPSC and its modulation

by classic neurotransmitters ACh and DA in the nAcb remain largely enigmatic. The present thesis attempts to provide some answers to these questions.

## 2. Anatomical and functional characteristics of the nucleus accumbens

Because of its similarities including cytoarchitecture, neurochemistry and afferent and efferent connections with the dorsal striatum, the nAcb is usually considered as a ventromedial extent of the striatum or the 'ventral striatum" (Heimer and Wilson, 1975; Heimer et al., 1997; Swanson and Cowan, 1975). Based on studies of its connectivity and distribution of neurotransmitters and chemical markers, the nAcb can be divided into two territories (Groenewegen and Russchen, 1984; Heimer et al., 1991; Brog et al., 1993; Zahm and Heimer, 1993). The portion of the nAcb surrounding the anterior commissure is known as the core where enkephalin and opioid receptors are rich, and strong immunoreactivity for calcium-binding protein, calbindin D28k (CaB) is found. The medio-ventral region of the nAcb is called the shell in which dense concentrations of substance P (SP), dynorphin, and tyrosine hydroxylase (TH) overlaping enkephalin- or CaB-poor zones are exhibited (Zahm and Brog, 1992; Jongen-Relo et al., 1994).

[**Figure 1.** The anatomical location of the nAcb]

### 2.1. Composition of the nucleus accumbens

#### 2.1.1. Innervations and projections

The major afferents of the nAcb arise primarily from limbic structures including the prefrontal cortex (PFC), hippocampus, basal amygdaloid complex and midline thalamic nuclei (Groenewegen et al., 1982, 1987; Jayaraman, 1985; Kelley and

Figure 1.

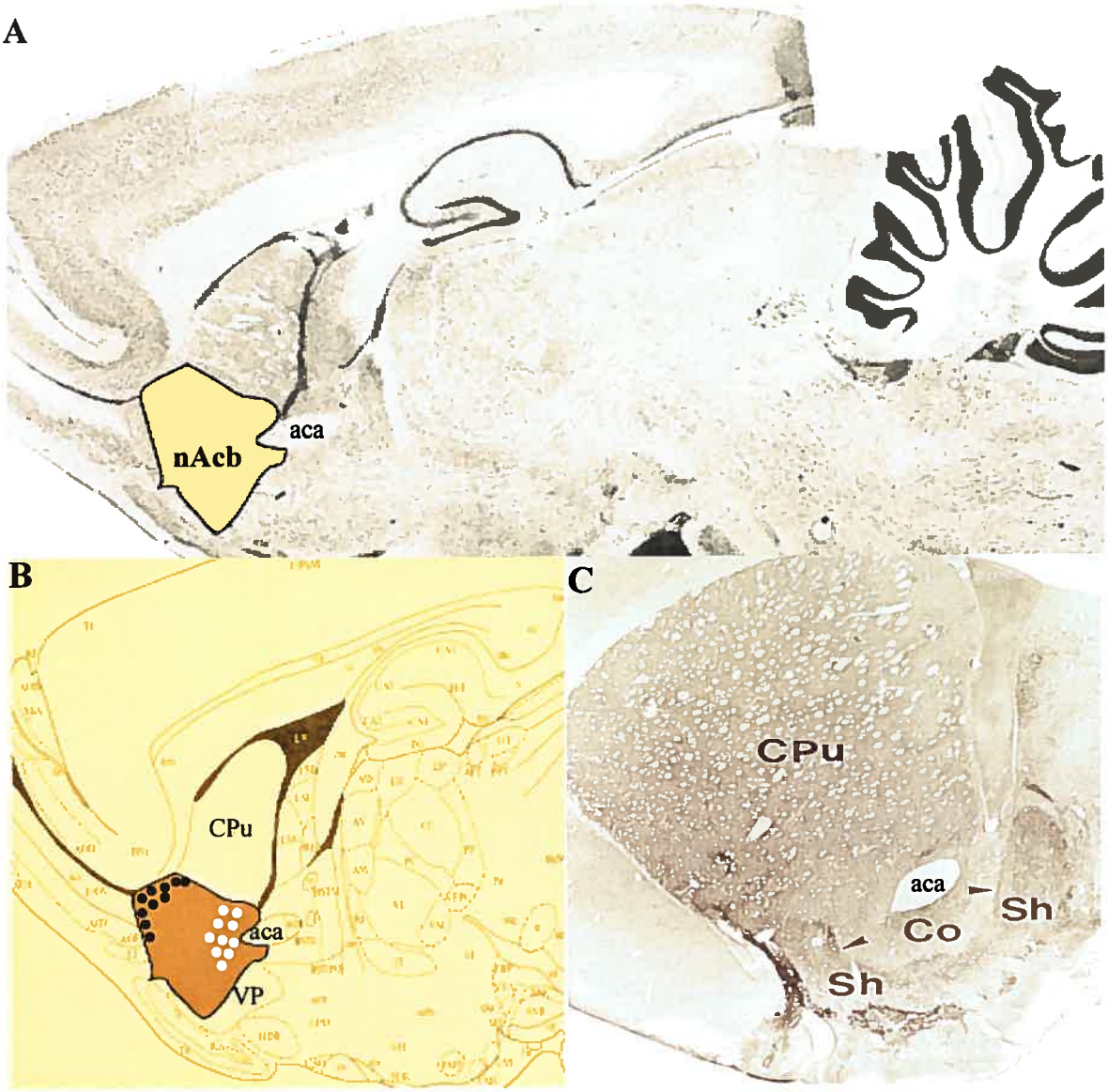


**Figure 1. An anatomical indication of the nAcb and its subterritories**
**A.** A parasagittal section of rat whole brain. **B.** A drawing shows mainly parts of forebrain to match the sagittal section. nAcb, nucleus accumbens (highlight areas); aca, anterior commissure; VP, ventral pallidum; CPu, caudate-putamen. The circles filled with white color indicate the sites where recordings were done in nAcb. The circles filled with black color show the places in which the stimuli were given for evoked responses. **C.** Coronal section through the forebrain of the rat showing SP immunoreactivity. Note that a border between the core(Co) and the dorsally adjacent main part of the striatal complex, CPu can not be identified, whereas the border between the Co and the shell(Sh) is distinct (arrowheads). (Paxions and Watson, 1986; De Olmos and Heimer,1999).

Domesick, 1982; Kelley and Stinus, 1984; Kelley et al., 1982; Krayniak et al., 1981; Newman and Winans, 1980; Phillipson and Griffiths, 1985; Meredith et al., 1990). These inputs are all thought to be glutamatergic. The nAcb also receives inputs from the ventral pallidum, dopaminergic VTA, serotonergic median raphe nucleus, and the noradrenergic cell group located in the nucleus of the solitary tract (Groenewegen et al., 1987; Brog et al., 1993; Berendse et al., 1992). The output of the nAcb is GABAergic and is primarily directed to the ventral pallidum (Hakan et al., 1992; Yang and Mogenson, 1985; Zahm and Heimer, 1990), which is involved in the activation of voluntary movements (Heimer et al., 1994; Swerdlow and Koob, 1987). This input-output organization suggests that the nAcb somehow provides a centre for limbic integration with motor systems driven by the ventral pallidum (Beninger et al., 1983; Lopes da Silva et al., 1984; Mogenson et al., 1980). In addition, the nAcb also projects to the VTA and the medial part of the substantia nigra pars compacta (Heimer et al., 1991; Mogenson et al., 1983; Nauta et al., 1978; Swanson and Cowan, 1975).

The core of the nAcb has been reported to receive its main cortical input from the prelimbic PFC (Brog et al., 1993; Sesack et al., 1989; Berendse et al., 1992; Montaron et al., 1996), and dorsal subiculum (Brog et al., 1993). It also appears to project to the dorsal portion of the ventral pallidum (Zahm and Heimer, 1990; Heimer et al., 1991). The shell receives its major inputs from the infralimbic PFC and the ventral subiculum (Kelley and Domesick, 1982; Brog et al, 1993; Yang and Mogenson, 1984; Sesack and Picel, 1990; Aylward and Totterdell, 1993) and sends projections to the ventro-medial part of the ventral pallidum.

[**Figure 2.** Diagram with inputs and outputs of the nAcb]

Figure 2.

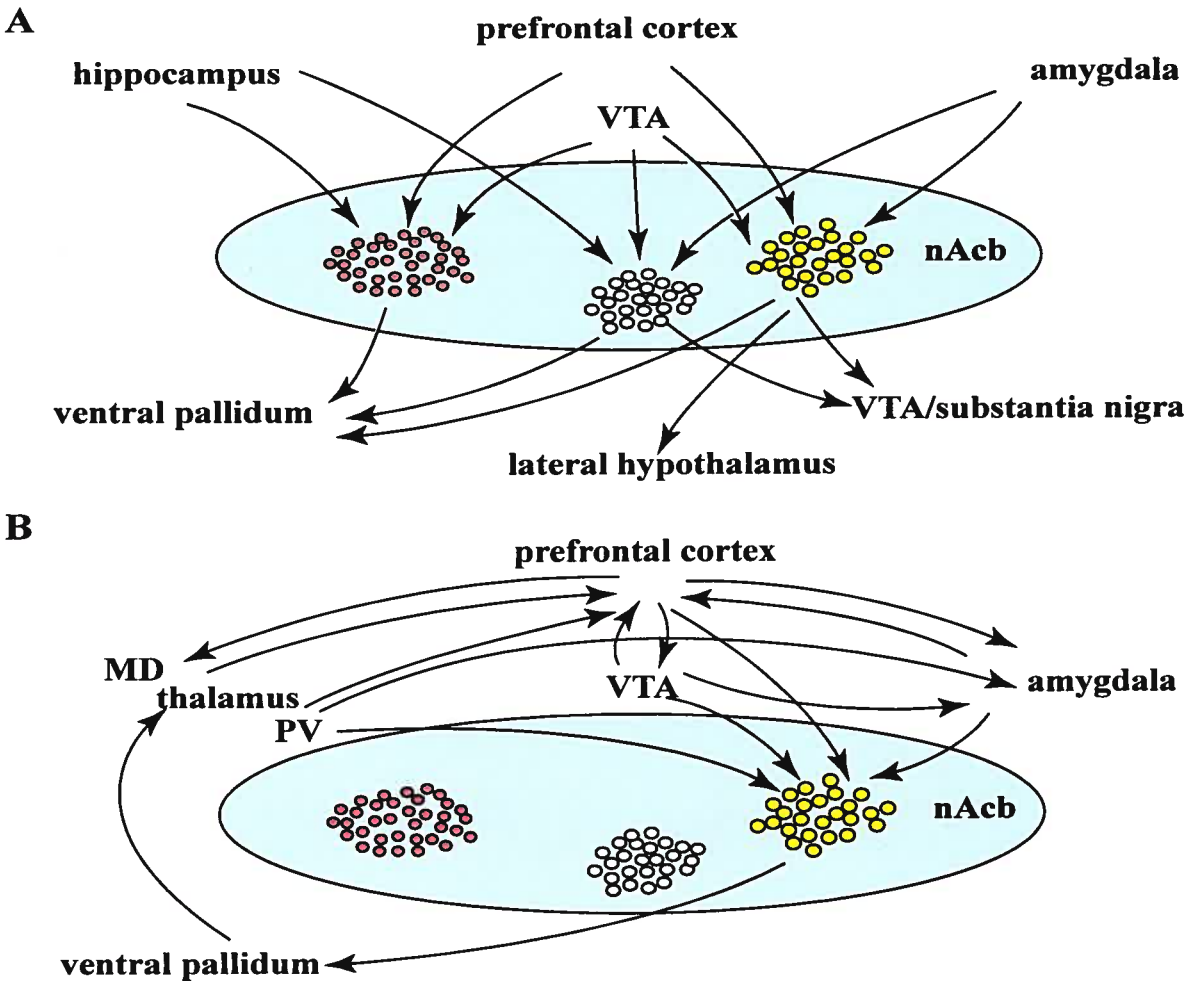


**Figure 2. Diagram with inputs and outputs of the nAcb**

Schematic representation of the input-output relationships of clusters of neurons in the nAcb. **A.** Different clusters of neurons (ensembles?) receive different combinations of converging inputs and project to distinct targets, including the ventral pallidum, the lateral hypothalamus, and the ventral mesencephalon. **B.** The various (limbic) cortical and subcortical structures that project to the nAcb are strongly and, in a number of cases, reciprocally interconnected. Note that the nAcb, via the ventral pallidum and the mediodorsal thalamic nucleus is involved in a closed thalamocortical-basal ganglia loop. nAcb, nucleus accumbens; MD, mediodorsal thalamic nucleus; PV, paraventricular thalamic nucleus. (Groenewegen et al., 1999).

### 2.1.2. Neuron types

As described in series of remarkable studies of the neostriatum (Bolam et al., 1984; Kawaguchi, 1992, 1993; Kawaguchi et al., 1989, 1990), the nAcb also consists of five different types of neurons in accordance with the neurochemical, morphological and physiological characteristics (Chang and Kital, 1985; Hedreen, 1981). Two types of the cells have been described with some details (Sesack and Pickel, 1990; O'Donnell and Grace, 1993). They are the GABAergic medium spiny (MS) neurons, which are the only identified projection neurons in the nAcb, and the large aspiny (LA) neurons, which are cholinergic interneurons. In addition to MS neurons, two other types of GABAergic interneurons have been identified: the fast spiking (FS) neurons and calretinin containing neurons. A third type, identified as the low threshold spike (LTS), which could also be GABAergic, contains somatostatin, neuropeptide Y and NO as co-transmitters.

#### 2.1.2.1. MS neurons

The principal neurons in the nAcb are the projection MS neurons (8-15 μm), which make up approximately 95% of all neurons (Chang and Kital, 1985). MS neurons appear to use GABA as one of their primary neurotransmitters (Fisher et al., 1986) along with several peptides as co-transmitters such as SP and neurotensin (Penny et al., 1986). MS neurons are by far the most frequently encountered cell type during physiological recordings and are recognizable by their strikingly large inward rectification during application of hyperpolarizing current pulses and their low RMP (around –80 mV). When recorded *in vivo*, MS neurons show a pattern of spontaneous activity consisting of long periods of silence separated by brief episodes of firing. Intracellular recordings *in vivo* in the nAcb (O'Donnell and Grace, 1995; Yim and Mogenson, 1988; Finch, 1996)

have shown that the silent and active episodes correspond to two different stable states of the membrane potential 10-20 mV apart: a hyperpolarized silent state around -80 mV and a depolarized active state around -60 mV. The shifts in membrane potential are relatively rapid (5 mV/s or more), of large amplitude, appear spontaneously *in vivo* and can last 100-500 ms. MS neurons fire only during the depolarized state with the spikes often occurring in bursts.

In nAcb slices maintained *in vitro*, the RMP of MS neurons is around -80 to -90 mV, corresponding to that of the hyperpolarized periods seen *in vivo* in normal animals and no depolarizing episodes are observed (Belleau and Warren, 1995, 2000). Because in the preparations *in vitro* neurons receive much less synaptic input than they do *in vivo*, it is unlikely that the membrane potential on MS neurons is maintained by barrages of inhibitory postsynaptic potential (IPSP). This suggests that the hyperpolarized state observed *in vivo* is not due to tonic inhibition, but rather because of a lack of excitation. Furthermore, Belleau and Warren (1995) found that $GABA_A$ receptor mediated IPSPs are depolarizing at RMP *in vitro*, showing that the hyperpolarized membrane potential of MS neurons is below the chloride ion equilibrium and may not result from $GABA_A$ mediated inhibition. Following lesion or reversible inactivation of subicular inputs *in vivo*, MS neurons remains in the hyperpolarized state. This suggests that the depolarized state requires the integrity of hippocampal inputs in the nAcb (O'Donnell and Grace, 1995).

#### 2.1.2.2. LA neurons

LA neurons are actually a group of giant cholinergic cells. This neuronal subtype has long been recognized as a separate cell type since it has a large somatic size (20-

60 μm) and an extensive aspiny dendritic tree that is much larger than those of MS neurons (Meredith et al., 1989; Kawaguchi, 1992; Zhou et al., 2002). An important step for their identification as interneurons was the discovery that they were the only source of ACh and choline acetyltransferase (ChAT) in the nAcb, since ventral and dorsal striatum inputs are devoid of any other cholinergic afferent (McGeer et al., 1971; Bolam et al., 1984). The RMP of LA neurons is more depolarized and closer to firing threshold than that of MS neurons. Consequently, they will fire more readily when injected with depolarizing current. Their firing patterns show little adaptation, but their firing frequency is limited by a large and long afterhyperpolarization (Beleau and Warren, 2000). Presumably, when they fire action potentials, they release ACh, which in turn modulate the excitability of the other neuronal elements of the nAcb by acting on ACh receptors. LA neurons are recognizable by their time-dependent rectification causing a large depolarizing sag in response to hyperpolarizing current pulses and the presence of a large and long duration spike afterhyperpolarization. The axonal fields of LA neurons are also more extensive than that of other accumbal neuronal elements and make most of their synapses with MS neurons (Izzo and Bolam, 1988; Phelps et al., 1985). In the dorsal striatum, LA neurons also receive convergent excitatory postsynaptic potentials (EPSPs) from cortical and thalamic stimulations (Wilson et al., 1990; Lapper and Bolam, 1992) that are probably mediated by both NMDA and AMPA types of glutamatergic receptors (Kawaguchi, 1992).

### 2.1.3. Synaptic framework in the nucleus accumbens

In the nAcb, DA and glutamate, which come from extrinsic sources, and ACh and GABA from local circuit neurons, are all capable of influencing the activity of accumbal

MS neurons. In the core region, the inputs onto spines and distal dendrites generally arise from extrinsic sources, whereas the synapses situated more proximally on dendrites or perikarya come from intrinsic sources (Meredith, 1999). By contrast, in the caudal medial shell MS neurons receive a mixture of intrinsic and extrinsic contacts, both distally and proximally. An important part of the intrinsic innervation of MS neurons is from other MS cells or from the local circuit neurons, such as LA neurons.

The primary asymmetrical input is from excitatory, presumably glutamatergic axons of cortical and thalamic origins. Inputs to both the core and shell regions arise from the amygdala and prefrontal area (Sesack and Pickel, 1992; Johnson et al., 1994). The lateral or medial entorhinal areas project primarily to the core, whereas the hippocampus innervates neurons primarily in the shell (Meredith, 1999; Meredith et al., 1993). Neurons in both midline and intralaminar thalamic nuclei project topographically to the core and shell, where they make asymmetrical contacts with dendrites and spines (Groenewegen et al., 1991; Meredith et al., 1993; Dube et al., 1988). Extrinsic inputs from dopaminergic centres, or intrinsic contacts such as those containing ACh provide additional but minor asymmetrical axospinous innervations to MS neurons. Glutamatergic nerve terminals make asymmetrical synaptic contacts with MS neurons, and asymmetrical synaptic specializations occur most commonly on the heads of dendritic spines and symmetrical inputs, along dendritic shafts, at the necks of spines, and on perikaryal membranes (Bolam, 1984; Meredith et al., 1993).

MS neurons also represent the main synaptic target of LA neurons (Graybiel, 1990; Izzo and Bolam, 1988). Cholinergic nerve terminals frequently form symmetrical synapses on their perikarya, dendrites and spines of MS neurons (Meredith and Chang,

1994), whereas the DA terminals are always found in a position proximal to those with glutamate, and, as such, are effective in gating signals from widely separated cortical areas. However, ultrastructural studies of cholinergic neuron innervation in the nAcb also suggest that ACh might regulate the release of other transmitters via presynaptic mechanisms, through a non-junctional or volumic mode of transmission (Contant et al., 1996).

The converging projections from glutamatergic and dopaminergic sources have been shown to synapse concurrently on dendrites of the same MS output neurons and axo-axonal juxtaposition between converging terminals has also been found (Bouyer et al., 1984; Totterdel and Smith, 1989; Sesack and Pickel, 1990). Neuroanatomical investigations demonstrate clearly that most dopaminergic afferents end directly on the MS neurons (Freund et al., 1984), although cholinergic interneurones do also receive some dopaminergic inputs (Chang, 1988; Kubota et al., 1987).

[**Figure 3.** A diagram with synaptic organization]

## 2.2. Membrane properties of MS neurons during postnatal development

The morphological and functional maturation of the nAcb probably depends on the interaction between the maturation of its neuronal elements and its innervation by extrinsic glutamatergic and other neuromodulatory inputs. It is likely that the disturbance of one or the other of these elements during a critical developmental period could lead to pathological states (Lipska et al., 1993, 1998; Weinberger and Lipska, 1995). Recent findings by Belleau and Warren (2000) revealed that around the time of birth and during the first postnatal weeks, the membrane and firing characteristics of MS neurons are quite

Figure 3.

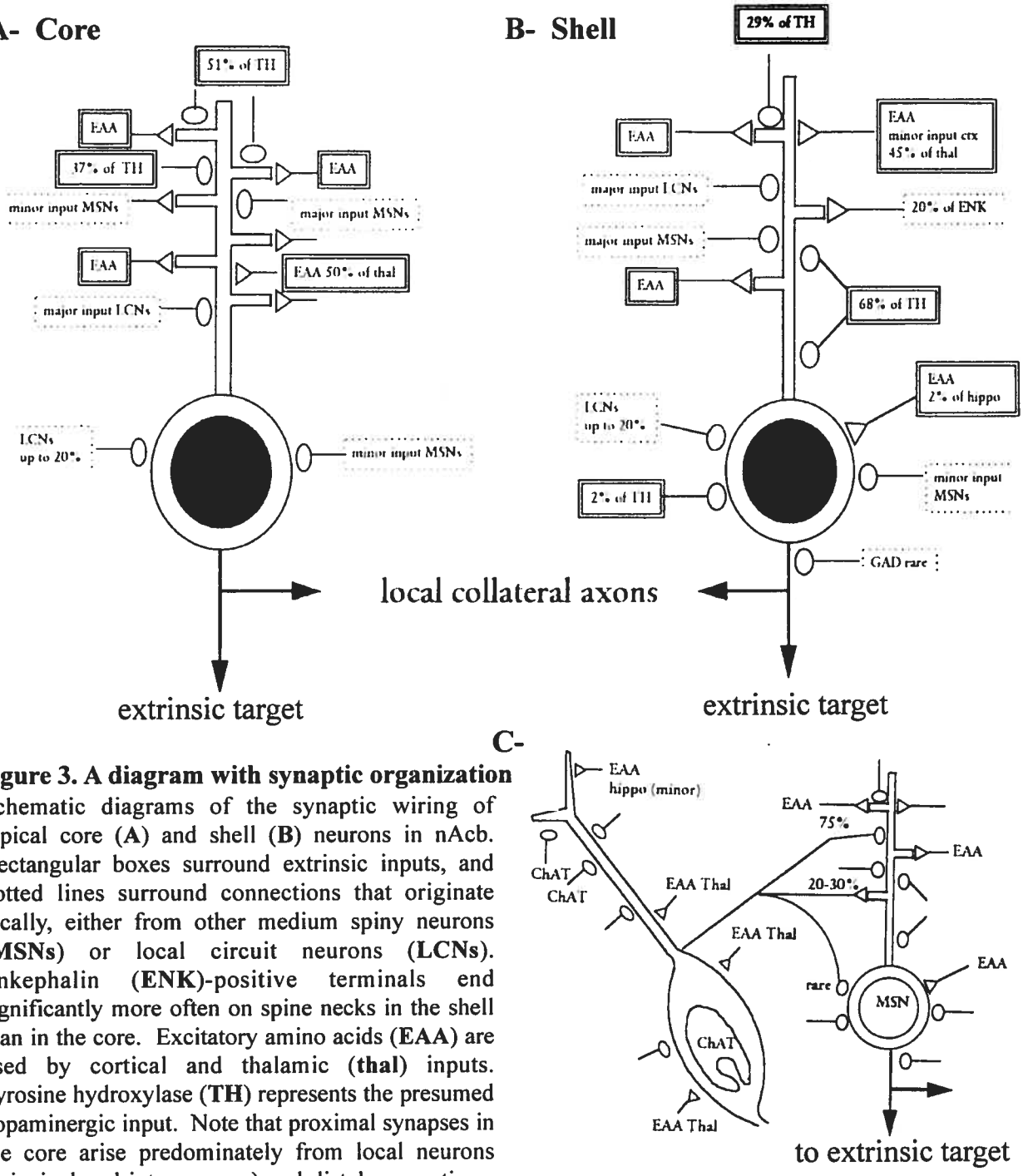


**Figure 3. A diagram with synaptic organization** Schematic diagrams of the synaptic wiring of typical core (**A**) and shell (**B**) neurons in nAcb. Rectangular boxes surround extrinsic inputs, and dotted lines surround connections that originate locally, either from other medium spiny neurons (**MSNs**) or local circuit neurons (**LCNs**). Enkephalin (**ENK**)-positive terminals end significantly more often on spine necks in the shell than in the core. Excitatory amino acids (**EAA**) are used by cortical and thalamic (**thal**) inputs. Tyrosine hydroxylase (**TH**) represents the presumed dopaminergic input. Note that proximal synapses in the core arise predominately from local neurons (principal and interneurons) and distal connections. ChAT-immunoreactive neurons, which are presumably cholinergic, receive cortical inputs (EAA) onto distal small dendrites but thalamic also (EAA) terminals proximally on the cell body or proximal dendrites. ChAT-positive endings contact other ChAT-positive dendrites. The cholinergic interneurons also contact the dendrites of MSN (**C**). GAD, glutamate decarboxylase; hippo, hippocampus (Meredith, 1999).

different from those observed later. These characteristics changed rapidly during first 3 postnatal weeks, at which point they resemble those found in adults. Both whole-cell membrane resistance and membrane time constant decreased more than four-fold during this period. During the first postnatal week, the current-voltage relationship of all encountered MS neurons was linear over a wide range of membrane potentials above and below RMP. Through the second postnatal week, the proportion of neurons displaying inward rectification in the hyperpolarized range increased steadily. After P15, all recorded MS neurons displayed significant inward rectification. At all ages, inward rectification was blocked by extracellular cesium and tetra-ethylammonium (TEA) but not by 4-aminopyridine (4-AP), suggesting that inward rectification was mediated by the same currents in young and mature MS neurons. MS neurons fired single and repetitive $Na^+/K^+$ action potentials as early as P1. Spike threshold and amplitude remained constant throughout development in contrast to spike duration, which decreased significantly over the same period. Depolarizing current pulse from rest showed that immature MS neurons fired action potentials more easily than their older counterparts.

The results suggest that young and adult nAcb MS neurons integrate excitatory synaptic inputs differently because of differences in their membrane and firing properties. These findings provide important insights into signal processing within nAcb during this critical period of development (Belleau and Warren, 2000).

Characteristics such as absence of inward rectification and consequent higher input resistance have also been demonstrated in cat MS neurons in dorsal striatum during the neonatal period (Cepeda et al., 1991). The maturation of these properties has a similar time course in both species, becoming essentially adult-like by the end of the third

postnatal week. Tepper and colleague (1998) found that the proportion of neurons that exhibited inward rectification increased steadily throughout postnatal development and reached a plateau by the end of the third postnatal week but still had not reached adult levels by the fifth postnatal week in rat striatum. The investigations from various groups have suggested that $K^+$ currents were responsible for inward and outward rectification in MS neurons (Nisenbaum et al., 1994; Nisenbaum and Wilson, 1995; Belleau and Warren, 2000). In addition, the mean RMP of MS neurons in the nAcb are –60 mV during the first postnatal week and below –80 mV after P21 (Belleau and Warren, 2000).

Compared to adults, membrane input resistance of MS neurons in neonatal rats is higher in the nAcb. However, input resistance decreased with postnatal development in MS neurons in the nAcb is closely correlated with inward rectification. The concomitant decrease in input resistance with age implies an increase in ion channel density. Cells displaying inward rectification display significantly more negative RMP than that of neurons lacking inward rectification (Belleau and Warren, 2000).

Despite their more depolarized membrane potential, no spontaneous activity in MS neurons in neostriatum in younger animals has been observed *in vitro* (Tepper et al., 1998; Napier et al., 1985; Tepper and Trent, 1993).

Discrete up and down states, as described in adult MS neurons *in vivo* (O'Donnell et al., 1999; Stern et al., 1997; Wilson and Kawaguchi, 1996) are absent in young neonates. A nominal condition for the appearance of membrane potential bistability in MS neurons is the presence of a more negative RMP such as that found *in vitro* in mature MS neurons (Tepper et al., 1998). The functional differences between young and mature

MS neurons could be important throughout a period during which activity-dependent development and stabilization of synaptic inputs is probably occurring in the nAcb. The nAcb receives putative excitatory glutamatergic input from various sources that are not fully developed at birth, so the nAcb is likely to complete its development in parallel with those structures (Belleau and Warren, 2000).

**3. An overview of ionotropic glutamate receptors and their properties**

Glutamate receptors mediate most of the excitatory synaptic transmission and play a crucial role in synaptogenesis and formation of neuronal circuitry as well as in synaptic plasticity including LTP and LTD. However, excessive activation of glutamate receptors might induce excitotoxic neuronal cell death and is also thought to contribute to neurodegeneration following a wide range of neurological insults including ischemia, trauma and epileptic seizures (Lipton and Rosenberg, 1994; Ozawa at al., 1998). The glutamate receptors are divided into two distinct groups, ionotropic and metabotropic receptors (Nakanishi, 1992; Seeburg, 1993; Hollmann and Heinemann, 1994). The ionotropic receptors are further subdivided into three groups: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), kainate (KA) and N-methyl-D-aspartate (NMDA) receptor channels on the basis of agonist specificities. However, since neither agonist nor antagonist clearly distinguished between AMPA and KA receptors in earlier time, they were often collectively referred to as non-NMDA receptors. AMPA receptors mediate the majority of fast excitatory synaptic transmissions (Hollmann and Heinemann, 1994; Borges and Dingledine, 1998; Dingledine et al., 1999). KA receptors contribute to postsynaptic responses at excitatory synapses and can also modulate presynaptic neurotransmitter release at some synapses (Frerking and Nicoll, 2000), whereas NMDA

receptors are crucial for the induction of specific forms of synaptic plasticity and play important roles in modulating synaptic strength, cell death and in several neuropsychiatric disorders (Hollmann and Heinemann, 1994; Dingledine et al., 1999; Malenka and Nicoll, 1999). The metabotropic receptors are coupled to G-proteins, and regulate the production of intracellular messengers (Ozawa et al., 1998).

### 3.1. Glutamatergic receptors-mediated EPSCs

At most central synapses, both AMPA and NMDA receptors are activated during synaptic transmission. Several lines of evidence suggest that AMPA and NMDA receptors are co-localized and commonly activated by glutamate liberated into the synaptic cleft (Jones and Baughman, 1991; Clements et al., 1992). EPSCs commonly have both AMPA and NMDA receptor-mediated components. The AMPA receptor-mediated EPSC (AMPAR-EPSC) has rapid kinetics of channel gating, whereas NMDA receptor-mediated EPSC (NMDAR-EPSC) has much slower rise and decay times relative to the AMPAR-EPSC (Hestrin et al., 1990; Lester et al., 1990; Keller et al., 1991). According to these different kinetics, it is believable that the AMPA receptor has a relatively low affinity for glutamate and becomes unbound very quickly after the clearance of the transmitter, whereas the NMDA receptor has much higher affinity, resulting in prolonged binding during which the channel can open repeatedly (Hestrin et al., 1990; Lester et al., 1990; Lester and Jahr, 1992).

### 3.2. AMPA receptors

AMPA receptors are ligand-gated channels usually considered to be permeable to $Na^+$ and $K^+$ and mediate fast excitatory synaptic transmission in central neurons

(Hollmann and Heinemann, 1994). AMPA receptors are encoded by four genes designated GluR1 through GluR4 and exist in $Ca^{2+}$-impermeable and $Ca^{2+}$-permeable forms. AMPA receptors assembled from GluR1, GluR3 and GluR4 alone or in combination are permeable to $Ca^{2+}$ and have doubly rectifying current-voltage relationships. The presence of GluR2 subunits render heteromeric AMPA receptor $Ca^{2+}$-impermeable (Hollmann et al., 1991, 1989; Verdoorn et al., 1991; Nakanishi, 1992; Seeburg, 1993; Hollmann and Heinemann, 1994). GluR2 subunits forming channels with other GluR subunits are $Ca^{2+}$-impermeable and electrically linear or outwardly rectifying (Pellegrini-Giampietro et al., 1997). The dominance of the GluR2 subunit in determining permeability to $Ca^{2+}$ and other divalent ions is attributed to the presence of a positively charged arginine in place of a glutamine residue within the M2 domain (Hume et al., 1991; Burnashev et al., 1992). Thus, the GluR1 and GluR3 to GluR2 ratio may be taken as a predictor of formation of $Ca^{2+}$-permeable AMPA receptors (Pellegrini-Giampietro et al., 1992). Different studies have demonstrated marked developmental changes in glutamate receptor subunit expression in rat brain (Pellegrini-Giampietro et al., 1991; Standley et al., 1995). In the neocortex, striatum and cerebellum, the GluR1+GluR3/GluR2 ratio is high at early postnatal stages and decreases monotonically with age, suggesting that a larger proportion of $Ca^{2+}$-permeable channels is formed during early neonatal than during adult life. In the hippocampus, the ratio increases from P7-P21, after which time it declines. Thus, the synthesis of GluR2 could provide a developmental mechanism regulating $Ca^{2+}$ permeable AMPA receptors at crucial times (Pellegrini-Giampietro et al., 1997).

2,3-benzodiazepines act as AMPA receptor-selective antagonists. Of these compounds, the drug GYKI53655 stands out as the most selective (Paternain et al., 1995; Wilding and Huettner, 1995). Quinoxalinediones such as 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 6,7-dinitroquinoxaline-2,3-dione (DNQX) are potent competitive antagonists at non-NMDA receptors (Honoré et al., 1988). In addition, 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonmide (NBQX) is a potent, selective and competitive AMPA receptor antagonist.

### 3.2.1. AMPA subunits and flip or flop isoforms during development

Each of the GluR1-GluR4 subunits exists in two different forms, "flip" and "flop", created by alternative splicing of a 115-base pair region immediately preceding the M4 segment (Sommer et al., 1990). Developmental and regional differences in expression of the alternative splice variants, flip and flop, have been demonstrated using *in situ* hybridization histochemistry (Sommer et al., 1990; Monyer et al., 1991).

AMPA receptor subunits are expressed predominantly in the flip form in embryonic brains. The flop form is expressed at low levels prior to P8, and gradually increases throughout the brain, reaching adult levels by P14, and then co-expresses with the flip form in several structures. Thus, excitatory neurotransmission in the adult brain appears to be mediated mainly by AMPA receptors carrying the flop module. AMPA receptors of the flip form are more resistant to desensitization than those of the flop form (Partin et al., 1995; Fleck et al., 1996), whereas receptors of the flop form show a faster desensitization rate than those with the flip form (Mosbacher et al., 1994). Recently, Seifert et al. (2000) have observed that the lowest sensitivity of AMPA receptors to KA

and NBQX began at P18, and suggested that these changes reflect a lower abundance of GluR1 at that developmental stage. A decrease of potentiation of receptor currents by cyclothiazide (CTZ), a selective AMPA receptor modulator to distinguish functionally between flip/flop variants, an acceleration of the recovery from CTZ potentiation and a faster and more complete desensitization of glutamate-evoked currents suggest an up-regulation of flop splice variants with increasing age in P3-45 rats in hippocampal CA1 neurons (Seifert et al., 2000). Several lines of evidence suggest that a reduction in flip form expression is likely to explain the developmental changes of AMPA receptor kinetics (Lawrence and Trussell, 2000).

### 3.3. KA receptors

The KA receptors are encoded by two gene families, KA1/KA2 and GluR5-7. Both of which have significant structural homology to AMPA receptors, GluR1-4 (Contractor et al., 2000). KA receptors that contain edited GluR5 subunits display a significantly reduced $Ca^{2+}$ permeability, a linear or slightly outwardly rectifying current voltage relationship and a single low conductance as well as a highly significant increase in the permeability to chloride ions (Chittajallu et al., 1999). KA receptors contribute to the EPSCs in response to glutamate and have been proposed to modulate synaptic transmission through an inhibitory presynaptic action (Frerking and Nicoll, 2000; Frerking et al., 2001). In the nAcb, functional KA receptors are abundantly expressed and can be activated by exogenous application of KA, but they do not directly participate in glutamatergic synaptic transmission evoked by electrical stimulation of cortical afferent fibers in the MS neurons. Activation of KA receptors in the nAcb inhibits

excitatory synaptic transmission including AMPAR- and NMDAR-EPSCs via a presynaptic mechanism (Casassus and Mulle, 2002; Crowder and Weiner, 2002).

Selective antagonists for KA receptors including NS-102, GYKI52466, GYKI 53655, LY293558 and LY294486 are providing novel pharmacological tools to allow the differentiation not only between AMPA and KA receptors but also between individual KA receptors comprising or containing GluR5 subunits (Chittajallu et al., 1999).

### 3.4. NMDA receptors

The NMDA receptor is a heteromeric protein complex constituting a cationic channel and several modulatory sites. It is a ligand-gated and voltage-dependant channel, which is highly permeable to $Ca^{2+}$. NMDA receptors are characterized by voltage-dependent block by $Mg^{2+}$, and at RMP it remains largely blocked by $Mg^{2+}$. In addition to the membrane depolarization required to remove the $Mg^{2+}$ block, NMDA receptors require the simultaneous binding of both glutamate and the co-agonist glycine for efficient gating (Ravenscroft and Brotchie, 2000). Ionic currents through the receptor only occur when the neuronal membrane is depolarized (Mayer et al., 1984; Nowak et al., 1984) with a high permeability to $Ca^{2+}$ (MacDermott et al., 1986; Mayer and Westbrook, 1987) and slow gating kinetics, i.e., NMDAR-synaptic transmission occurs slowly and lasts for a prolonged period compared to AMPAR-currents (Lester et al., 1990). During synaptic transmission, EPSC generated by NMDA receptor activation occurs with slow rise and an exceptionally slow decay time, which exceeds that of AMPAR-EPSC by several orders of magnitude. NMDA channels first open about 10ms after glutamate is

released into the synaptic cleft, and then last hundreds of milliseconds until glutamate unbinds from the receptor (Behe et al., 1999; Dzubay and Jahr, 1996).

In external medium containing physiological concentrations of $Mg^{2+}$ (~1 mM), the NMDAR-current is maximal between –20 and –30 mV, and is reduced at more hyperpolarized potentials despite the increased electrical driving force. The inward current is negligible at –80 mV, and the I-V relationship of the NMDA response thus exhibits a clear negative slope conductance between –80 and –30 mV. The negative slope conductance is eliminated by removing $Mg^{2+}$ from the external solution (Mayer et al., 1984; Nowak et al., 1984).

### 3.4.1. Glycine, a co-agonist

Glycine is a co-agonist of the NMDA receptor (Johnson and Ascher, 1987). The NMDA response is markedly potentiated by glycine in cultured central neurons. NMDA responses were not detected without glycine in the external solution. This implies that glycine is not simply a strong potentiator of the NMDA response, but is absolutely necessary to enable the NMDA receptor channel to enter the open state, it thus plays a role as a co-agonist. However, NMDA responses are still detectable because of the presence of endogenous glycine. 7Cl Kyn could almost completely abolish NMDA responses by competitively displacing glycine from its binding site (Kemp et al., 1988; Vyklicky et al., 1990).

### 3.4.2. NMDA receptor subunits and developmental regulation

The NMDA receptor consists of a NR1, NR2 and two NR3 subunits. The NR2 includes NR2A, NR2B, NR2C and NR2D subunits (Hollmann, 1999; Moriyoshi et al., 1991; Das et al., 1998; Ikeda et al., 1992; Kutsuwada et al., 1992; Meguro et al., 1992; Monyer et al., 1992; Ishii et al., 1993). All NMDA receptors appear to function as heteromeric assemblies composed of multiple NR1 subunits in combination with at least one type of NR2. The NR3 subunit does not form functional receptors alone, but co-assembles with NR1/NR2 complexes (Das et al, 1998; Perez-Otano et al, 2001).

The time course of decay of NMDAR-EPSCs and the apparent affinity of the receptors for glutamate are both strongly influenced by the identity of the NR2 subunits involved. The functional properties of NMDA receptor channels such as the degree of voltage-dependent $Mg^{2+}$ block and deactivation kinetics depend on which of the four NR2 is assembled. Diheteromeric NMDA receptors containing NR2A or NR2B subunits generate 'high-conductance' channel openings with a high sensitivity to blockade by $Mg^{2+}$, whereas NR2C- or NR2D-containing receptors give rise to 'low-conductance' openings with a lower sensitivity to extracellular $Mg^{2+}$. The deactivation times follow the sequence: NR2A<2C=2B<<2D. Thus, a brief application of glutamate onto NR1/NR2A assemblies generates a macroscopic current with a deactivation time constant of tens of ms, compared with several seconds for NR1/NR2D receptors (Monyer et al., 1994; Wyllie et al., 1998; Vicini et al., 1998).

The NR2 subunits are regulated developmentally in rodent brains (Watanabe et al., 1992; Monyer et al., 1991). NR2B and NR2D subunits predominate in the neonatal brain, but over the course of development these are supplemented with, or replaced by NR2A and in some regions by NR2C subunits (Monyer et al., 1994; Akazawa et al.,

1994). At embryonic stages, the NR2B subunit is found in most brain regions, whereas the NR2D subunit is present in the diencephalon and brainstem. Soon after birth, NR2A mRNA is found in most regions, whereas NR2C appears later and is predominant in the cerebellum (Monyer et al., 1994; Akazawa et al., 1994). A general trend shows that the contribution of NR2B subunit is decreasing during development, which is associated with an increasing contribution of NR2A-containing NMDA receptors to synaptic current. Additionally, when NR2A is expressed, it almost always co-expresses with NR2B at P3-P9 ages in some brain regions. Neurons expressing NR2A subunit mRNA have faster NMDAR-EPSCs than cells not expressing this subunit, regardless of postnatal age. Expression of NR2A subunit mRNA in cortical neurons at even low levels seems sufficient to alter the NMDA receptor time course. Generally, the proportion of cells expressing NR2A and displaying fast NMDAR-EPSCs increase developmentally (Flint et al., 1997).

### 3.4.3. Components of subunits determine properties of NMDA receptors

$Mg^{2+}$ inhibition of NMDA currents (Nowak et al., 1984) is almost exclusively displayed by receptors containing NR1/NR2A or NR1/NR2B subunits (Kutsuwada et al., 1992; Monyer et al., 1992). NMDA receptors containing NR2C exhibit a low sensitivity to $Mg^{2+}$, which would be expected to allow these NMDA receptors to operate at more negative membrane potentials than conventional NR2A/B-containing receptors. This difference may explain, in part, the ability of antagonists with moderate selectivity for NR2A/B- or NR2C/D-containing receptors to differentially block LTP and LTD in the hippocampus (Hrabetova et al., 2000). In external solution containing 1 mM $Mg^{2+}$, the current response was largest at –25 mV in the NR2A/B two-receptor channels, whereas it

was around –45 mV in the NR2C/D two receptor channels. Furthermore, the blockage by $Mg^{2+}$ on the inward current is much stronger in the range between –25 and –80 mV for the former than the latter (Monyer et al., 1994). The main functional properties, such as the sensitivity to $Mg^{2+}$ and ifenprodil, and the time course of synaptic current suggest that most receptors are composed of NR1 and NR2B subunits (Plant et al., 1997).

Recent studies found that NR1 subunits also strongly influence NMDA receptor properties. For example, the pH sensitivity of NMDA receptors is determined by the presence of exon 5 of the NR1 subunits. At physiological pH, splice variants that include exon 5 are fully active, whereas those lacking exon 5 are partially blocked (Traynelis et al., 1995). Most importantly, it has been shown that splicing of exon 5 can influence the deactivation properties of NMDA receptors (Rumbaugh et al., 2000). Unlike NR2A-containing receptors (Vicini et al., 1998), the deactivation time of recombinant NR2B-containing receptors is dependent on whether or not NR1 contains the exon 5 insert. The deactivation rate is roughly four times faster for NR1-1b/NR2B (exon-5-containing) receptors than for the NR1-1a/NR2B (exon-5-lacking) receptors. This observation may well be relevant to the change in time course of the NMDAR-EPSC decay that occurs at many synapses during development (Laurie and Seeburg, 1994).

A gradual replacement or supplementation of NR2B by NR2A during postnatal development has been implicated in the speeding of NMDAR-EPSC decay—a phenomenon often linked with the ability of neuronal circuits to exhibit experience-dependent synaptic plasticity (Constantine-Paton and Cline, 1998). For example, the NMDAR-EPSCs in the visual cortex are sensitive to NR2B-selective antagonists, ifenprodil, when the NMDAR-EPSC decay is slow at P3-P5. This sensitivity is lost by

P7 when the NMDAR-EPSC decays more rapidly. Therefore, to test EPSC sensitivity to ifenprodil has been a reliable indicator for NR2B subunit-containing NMDA receptors. Reversibly, NR2A/NR2B subunits ratio appears to be an indicator of NMDAR-EPSCs decay (Quinlan et al., 1999a).

Investigation suggests that native nAcb NMDA receptors are composed of NR1/NR2B and maybe to a lesser extent of NR1/NR2A subunits, although a combination of these three subunits is also possible (Chazot and Stephenson, 1997; Le Greves et al., 1997).

### 3.4.4. Single channel properties during development

The native NMDA receptor channel has ~50 pS conductance levels when open and ~40pS sublevels in various central neurons (Nowak et al., 1984; Jahr and Stevens, 1987; Ascher et al., 1988). Farrant and colleagues (1994) have shown that the single channel properties of the NMDA receptor in cerebellar granule cells markedly changes during early development. At an early stage (before P13), most openings were of the 50/40pS state. In contrast, the majority of channel openings (65%) were to the lower conductance state (~33/20pS) at P19-23. Expression studies have shown that both NR1/NR2A and NR1/NR2B NMDA receptors have ~50/40 pS openings (Stern et al., 1992; Tsuzuki et al., 1994), whereas NR1/ NR2C and NR1/NR2D receptor openings have lower conductance (~35/20 pS) (Stern et al., 1992; Wyllie et al., 1996). These results strongly suggest that changes in single channel properties of the NMDA receptor during early development is due to developmental changes in expressions of the NR2 subunits. These findings are consistent with the results from cerebellar granule cells in

which at P7 the single channel conductance of the NMDA receptor is predominantly ~50/40 pS. At P30, the low-conductance (~34/18pS) channels become dominant, and very few high-conductance channels are detected (Takahashi et al., 1996).

It is most likely that the high-conductance channels are produced predominantly by NR1/NR2B and NR1/NR2A combinations in immature and mature animals, respectively, since *in situ* hybridization studies have shown that NR2A is expressed relatively late postnatally whereas NR2B is expressed transiently during the earlier stage in cerebellar granule cells (Watanable et al., 1992; Monyer et al., 1994).

D-2-amino-5-phosphonovalerate (D-APV), 7-chlorokynurenic acid (7Cl Kyn), $Mg^{2+}$, and MK-801 are different NMDA receptor antagonists (Ozawa et al., 1998). Additionally, Ifenprodil and a group of related compounds, such as, Haloperidol and CP101, 606 are selective antagonists of NR2B-containing NMDA receptors, whereas PPDA is a competitive inhibitor of NR2C and NR2D subunits (Cull-Candy et al., 2001). Ifenprodil selectively block NR2B-containing NMDA receptors in a non-competitive, voltage-independent, activity-dependent manner (Constantine-Paton and Cline, 1998; Moriyoshi et al., 1991; Sugihara et al., 1992).

**4. Cholinergic receptors**

The neurotransmitter ACh is released from vesicles in presynaptic nerve terminals and influences functional and behavioral states through its actions at metabotropic muscarinic ACh receptors (mAChRs) and/or ionotropic nicotinic ACh receptors (nAChRs) (Guo and Chiappinelli, 2000). Five mAChR subtypes (M1-M5) have been identified by molecular cloning (Caulfield and Birdsall, 1998). Although a number of

subunits of neuronal nAChRs (α2-α9, β2-β4) have been cloned, and several subtypes of nAChRs are known in brain, a complete nomenclature has not yet been achieved due to the numerous combinations of these subunits that can form native nAChRs (Sargent, 1993). Responses mediated by nAChRs are always excitatory, occur rapidly, and are blocked by d-tubocurarine. In contrast, muscarinic responses can be either excitatory or inhibitory, depending on the mAChR subtype mediating the response and the type of G-protein to which the mAChR is coupled (Brown et al., 1997; Felder, 1995). These responses have longer latency of onset and can be blocked by atropine or scopolamine. In some brain regions, mAChRs are co-localized with nAChRs, suggesting that cholinergic modulation is complex in the CNS (Quirion et al., 1994). One important function of ACh receptors (AChRs) localized on or near presynaptic terminals is its role in modulating neurotransmitter release (Caulfield, 1993; Wonnacott, 1997).

### 4.1. Cellular localization of cholinergic receptors

#### 4.1.1. Muscarinic ACh receptors

M1 receptors are distinctly expressed in the nAcb (Mash and Potter, 1986; Blake et al., 1991; Kohler et al., 1995; Kushida et al., 1995; Adem et al., 1997). Immunocytochemistry and *in situ* hybridization studies of mAChRs in the neostriatum-including nAcb suggest that probably all neurons express mAChRs but different types of neurons appear to express different types of mAChRs. Studies agree that M1 mAChR is the most abundant, being detected in the soma of 78-85% neurons displaying the characteristics of MS neurons (Bernard et al., 1992; Hersch et al., 1994; Weiner et al., 1990). M4 receptors are found in a subset of putative MS neurons: all those containing

substance P and 39% of those containing enkephalin (Bernard et al., 1992), suggesting that a subset of MS neurons express only M1 receptors whereas others express both M1 and M4 receptors. At subcellular levels, immunocytochemically labeled M1 receptors were enriched in spiny dendrites, at postsynaptic densities and on a small number of axon terminals forming asymmetrical synapses. M4 receptor protein was also found on axon terminals that formed asymmetrical synapses (Hersch et al., 1994). These findings are interesting since neostriatal asymmetrical synapses are known to originate primarily from glutamatergic cortical, thalamic, and subthalamic afferents. This localization suggests the presence of postsynaptic mAChRs close to synapses using excitatory amino acids as their primary neurotransmitter.

Most somatostatin (putative LTS interneurons) and neurotensin containing neurons express M1 receptor gene but only a few express M4 receptor mRNA. In contrast to M1 receptor, M2 receptor protein was found in only 2.5% of neostriatal neurons and those had characteristics of LA neurons. Many of these were found on axon terminals, usually making symmetrical synapses with somata, dendritic shafts and spines whereas a minority contacted terminals forming asymmertrical synapses on spines or dendrites (Hersch et al., 1994). *In situ* hybridization studies showed that, in addition to M2 receptors (Bernard et al., 1992; Weiner et al., 1990; Yan and Surmeier, 1996), a large proportion of putative cholinergic neurons also contained M4 receptor mRNA while some may have contained M1 mRNA (Bernard et al., 1992; Yan and Surmeier, 1996). M3 receptor protein was only found in a distinct population of small dendrites and some axon terminals forming asymmetrical synapses. These findings indicate that mAChR proteins are concentrated postsynaptically on non-cholinergic and cholinergic neurons.

In addition, each receptor subtype is also found on presynaptic terminals making both asymmetrical and symmetrical synapses, suggesting that ACh probably modulate neurotransmitter release and that M2 receptors are the predominant muscarinic autoreceptor (Hersch et al., 1994). Other studies also suggested that ACh varicosities in the nAcb were presumably involved in a non-junctional or volumic mode of transmission (Contant et al., 1996).

### 4.1.2. Nicotinic ACh receptors

Both anatomical and neurochemical studies in the mid-1980s suggested that nAChRs were localized at presynaptic sites within the peripheral nervous system and CNS (Schwartz et al., 1984; Clarke et al., 1986; Vizi and Somogyi, 1989). Light and electron microscopic studies, using radiolabled nAChR ligands and immunological probes, revealed the presence of nAChR binding sites over the length of individual nerve fibers and provided evidence for the anterograde shipment of nAChRs to synaptic terminals (Clarke et al., 1986; Torrao et al., 1996; Wonnacott, 1997). Furthermore, studies looking at nAChR distribution following the destruction of presynaptic projections provided strong anatomical evidence for the localization of nAChRs on presynaptic terminals (Clarke et al., 1986; Torrao et al., 1996). These findings have recently received elegant confirmation in experiments using immuno-gold labeling of neuronal nAChRs to demonstrate that α7 subunits were located on presynaptic terminals within the PFC (Lubin and Aokie, 1998).

It is now clear that nAChR genes are expressed in neostriatal neurons, especially the α3 and β2 subunits, with a restricted and heterogeneous pattern (Wada et al., 1989).

The localization of nAChRs in presynaptic structures in the CNS has received important support from studies using synaptosomes preparations from a variety of brain regions indicating that nAChRs are present in presynaptic membrane not from only the nAcb but also other parts of CNS, including striatum, PFC, hippocampus, thalamic, and hypothalamic nuclei (Wonnacott, 1997).

#### 4.1.2.1 Subunit composition of functional nicotinic ACh receptors

With the binding of ACh or other agonists, nAChRs allow cations to flow through an intrinsic channel, generally resulting in depolarization of the neuron. To date, 13 different neuronal nAChR subunits (α2-α10 and β2- β4) have been identified (Le Novère and Changeux, 1995). The neuronal nAChR subunits can be divided into subfamilies based on sequence homology and phylogeny (Le Novère and Changeux, 1995), as well as pharmacological and physiological properties. α1-α6 combined with β2-β4 would comprise one family and α7-α8 would comprise the second. α9 has distinct pharmacological properties and is likely to be part of a third family. Functionally, nAChRs can be divided into those containing the β2 subunit which combine with different α subunits, forming receptor with the highest affinity for nicotine (Picciotto et al., 1995), and those containing the β4 subunit which also combined with various α subunits, forming receptors with 10-100 times lower affinity for nicotine (Luetje and Patrick, 1991). β4/α3 subunit-containing nAChRs are highly expressed in the peripheral nervous system and appear to be essential for fast synaptic transmission in the autonomic ganglia (Xu et al., 1999). In contrast, the subtypes of nAChR expressed most commonly in the brain are made of β2/α4 subunits and α7 subunits and bind to α-bungarotoxin (Flores et al., 1992; Hill et al., 1993; Wada et al., 1989; Zoli et al., 1995).

## 5. Muscarinic ACh receptors and synaptic transmission in the nucleus accumbens

Over the last ten years, the use of *in vitro* brain slice preparation to record single nAcb neuron and the availability of more selective pharmacological compounds to isolate the function of various neurotransmitter receptors have enabled researchers to understand the contribution of different transmitters in the generation and modulation of the neuronal activity in the nAcb.

### 5.1. Activities of muscarinic receptors modulate the function of MS neurons

Each identified neuronal population in the nAcb is under the direct control of the ACh neurons through the expression of mAChRs, in particular the M1 receptor (Adem et al., 1997; Kohler et al., 1995; Bernard et al., 1992). Of five subtypes of mAChRs, the M1, M3 and M5 receptors subtypes activate phospholipase C (PLC) that release inositol 1,3,5-triphosphate (IP3) and diacylglycerol (DAG) from membrane phospholipid (Berridge and Irvine, 1984). Conversely, the M2 and M4 receptor subtypes inhibit adenylate cyclase (AC) (Hulme, 1990). Application of muscarinic cholinergic agonists has been found to produce either excitatory or inhibitory effects on the MS neurons.

#### 5.1.1. Modulation of ionic conductance by activating muscarinic ACh receptors

Muscarine predominantly reduces inward rectifier conductance and causes membrane depolarization of MS neurons. These effects are competitively antagonized by pirenzepine, a selective M1 receptor antagonist, indicating involvement of the M1 receptor in nAcb MS neurons (Uchimura and North, 1990; Hsu et al., 1996). Additionally, carbachol can also act at M1 muscarinic receptors to reduce the membrane $K^+$ conductances and excite MS neurons (Hsu et al., 1996).

*In vitro* studies in rat corticostriatal slice preparations show that low concentration of muscarine which does not alter the membrane potential and input resistance of MS neurons, increases excitability by enhancing the membrane depolarization and inward current produced by the application of NMDA but not of AMPA (Calabresi et al., 1998). This facilitatory effect is not affected by TTX, suggesting a postsynaptic site of action, and is mimicked by neostigmine. This indicates that endogenous ACh may also enhance responses to NMDA. The pharmacological analysis of this phenomenon also suggests the involvement of M1 receptors. In fact, this facilitatory action has been antagonized by pirenzepine and mimicked by McN-A-343, a selective M1 receptor agonist. Interestingly, McN-A-343 also increases the duration of the glutamate-mediated EPSPs elicited by the stimulation of corticostriatal fibers. However, this occurs only in the absence of $Mg^{2+}$ ions from the external bathing solution, suggesting the involvement of a NMDA component in corticostriatal synaptic potentials. This provides further confirmation that M1 receptor activation might selectively exert a positive and modulatory role on NMDAR-potentials. Additionally, muscarinic agonists have been reported to decrease the responsiveness of dorsal striatal neurons to excitatory inputs (Dodt and Misgeld, 1986; Akaike et al., 1988; Malenka and Kocsis, 1988). In cultured rat dorsal striatal neurons, Akins et al. (1990) found that muscarinic agonists shifted the voltage-dependence of the A-current activation and inactivation towards more negative membrane potentials, in addition to increasing its peak conductance. Thus, in the presence of muscarinic receptor agonists at relatively hyperpolarized membrane potentials, this allowed the A-current to suppress excitatory inputs and further slow the discharge rate.

High-voltage-activated $Ca^{2+}$ current through striatal MS neurons appear to be another postsynaptic target of mAChR agonists. Current-clamp experiments performed on rat striatal slices show that the activation of mAChRs significantly reduces the duration of $Ca^{2+}$ dependent plateau potentials (Misgeld et al., 1986). More recently, whole-cell patch-clamp experiments with acutely isolated striatal neurons showed that mAChR agonists reduce $Ca^{2+}$ currents via two distinct signaling pathways depending on pertusis-toxin-insensitive G proteins and is responsible for the inhibition of L-type $Ca^{2+}$ channels (Howe and Surmeier, 1995).

### 5.2. Activation of muscarinic receptors modulate glutamate release

In the nAcb, the depression of excitatory synaptic transmission mediated by presynaptic mAChRs has also been demonstrated (de Rover et al., 2002; Pennartz and Lopes da sliva, 1994; Sugita et al., 1991). Stimulus-evoked EPSPs can reversibly be attenuated by muscarine or carbachol, and this action is completely antagonized by atropine or pirenzepine, indicating that the effect of muscarinic receptor agonist on EPSPs is mediated by M1 receptor. Carbachol induces no alteration of glutamate-evoked depolarization while suppressing EPSPs in the same neuron, suggesting the involvement of a presynaptic mechanism in muscarinic inhibition (Pennartz and Lopes da sliva, 1994).

The action of mAChRs activation on the release of excitatory and inhibitory transmitters has also been studied. Both glutamate- and GABA-mediated synaptic potentials are reduced in a dose-dependent manner by mAChR agonists. A heterogenous population of mAChRs seems to be involved in this cholinergic action on glutamatergic and GABAergic nerve terminals. Sugita et al. (1991) have shown that muscarine and

ACh inhibited the release of both excitatory amino acids and GABA through M3 and M1 receptors in the nAcb, respectively via distinct presynaptic mechanisms.

In the striatum, early studies *in vivo* demonstrated that iontophoretically applied ACh to striatal neurons produced both excitatory and inhibitory effects: ACh increased the spontaneous firing rate but reduced the EPSPs (Bernardi et al., 1976). Takagi and Yamamoto (1978) reported that cholinergic agonist or physostigmine exerted inhibitory effects on single unit activity induced by local stimulation of the striatum *in vitro* without affecting glutamate-induced firing activity. These extracellular experiments suggest that ACh and carbachol have presynaptic inhibitory effects on striatal neurons (Takagi and Yamamoto, 1978).

### 5.3. ACh release regulated by muscarinic ACh receptors in LA neurons

James and Cubeddu (1987) reported that muscarinic agonists limited ACh release in the dorsal striatum. Later, two different physiological actions of mAChR activation have been described on LA interneurons. Both actions resulted in the inhibition of LA neurons activity, confirming that ACh regulates its own release via the activation of muscarinic autoreceptors. The first action of mAChRs activation described in cholinergic interneurons is the inhibition of both N- and P-type $Ca^{2+}$ channels mediated by M2 receptors via the stimulation of a G-protein dependent intracellular pathway (Yan and Surmeier, 1996). As $Ca^{2+}$ channels take part in the regulation of neurotransmitter release, the inhibition of these currents might, at least partially, explain the inhibitory effect of ACh on its own release. More recently, the existence of a novel mechanism has been proposed to underlie the mAChR-mediated modulation of ACh release in the dorsal

striatum (Calabresi et al., 1998). Intracellular recordings from identified LA neurons *in vitro*, combined with focal stimulation, reveals a mAChRs dependent IPSP, suggesting that the release of endogenous ACh from presynaptic nerve terminals inhibits LA neuron activity by activating muscarinic autoreceptors located in their somato-dendritic region. This IPSP appears to be mediated by the opening of $K^+$ channels since it was blocked by barium and reversed at the $K^+$ equilibrium potential. This inhibitory effect also appears to be mediated by M2 receptors, as it is blocked by methoctramine, but not by pirenzepine. Furthermore, the membrane hyperpolarization mediated by nerve-terminal stimulation can be mimicked by the exogenous application of muscarine and oxotremorine, but not by McN-A343 (Calabresi et al., 1998).

**6. Activation of nicotinic ACh receptors facilitates synaptic transmission in the CNS**

It has been proposed that the role of nAChRs in the CNS is to primarily modulate synaptic transmission rather than to mediate it (Gray et al., 1996; McGehee et al., 1995). Several effects of nicotine in the brain appear to be mediated through the neuromodulatory potentiation of different neurotransmitters including norepinephrine, DA, GABA, serotonin, ACh, and glutamate (MacDermott et al., 1999; McGehee and Role, 1996; Wonnacott et al., 1989).

**6.1. Facilitation of glutamate release by presynaptic nicotinic ACh receptors**

Nicotinic facilitation of glutamate release has been reported in different areas of the CNS (Toth et al., 1993; McGehee et al., 1995; Guo et al., 1998; Radcliffe and Dani, 1998; Girod et al., 2000; Fisher and Dani, 2000; Reid et al., 2000; Schilstrom et al., 2000). Nicotine lifts extracellular glutamate levels in the nAcb (Reid et al., 2000) and the

dorsal striatum (Toth et al., 1993). DMPP, a specific nAChR agonist, has also been found to increase the frequency of CNQX-sensitive postsynaptic currents in CNS via presynaptic action on nAChRs located on glutamatergic terminals (Bordey, et al., 1996). In addition, nAChRs have been found expressing on glutamatergic terminals in olfactory bulb culture, where they have been shown to modulate glutamate release and subsequently modulate excitatory transmissions (Alkondon, et al., 1996). Studies have shown that nicotine not only enhances AMPAR-synaptic transmission (McGehee et al., 1995), but NMDAR-synaptic transmission (Aramakis and Metherate, 1998) as well in the CNS during postnatal development by presynaptic mechanisms. Several lines of evidence also suggest that ACh may directly or indirectly influence the excitability of neurons in the nAcb (Gauchy et al., 1991).

The addictive effects of nicotine are largely attributed to its efficacy in eliciting DA release in the nAcb whereas the purported effects of nicotine on memory, attention, and arousal may reside in its potent facilitation of glutamate release at hippocampal and habenula-interpeduncular synapses (Dani, 2003; Mansvelder and McGehee, 2002; Balfour, 2002; Vogt and Regehr, 2001; Shim et al., 2001; Radcliffe and Dani, 1998)

Characteristics of the presynaptic nAChRs that facilitate glutamate release implicate different nAChR subtypes (Gray et al., 1996; McGehee et al., 1995; Guo et al., 1998). The nicotine- and ACh-induced enhancement of evoked and spontaneous glutamate release often appears to be mediated by α7-containing receptors. These sites are gated by nanomolar concentrations of nicotine and are blocked by both MLA and αBgtX. Furthermore, rapid, repeated high-dose exposure of glutamatergic synapses to ACh could elicit a sustained potentiation of synaptic transmission (Girod et al., 2000).

The selective, pharmacological targeting of α7-containing nAChR subtypes is of particular importance in view of the reported decreases in cholinergic projections and α7-containing receptors in pathological conditions involving cognitive and/or attentional deficits, such as Aizheimer's disease and schizophrenia (Benowitz, 1996; Lindstrom, 1997; Lloyd et al., 1998).

**6.2. Facilitation of dopamine release by presynaptic nicotinic ACh receptors**

High density and affinity nAChRs are present on terminals of dopaminergic projections to the nAcb (Clarke and Pert, 1985). Recent studies have consistently demonstrated that nicotine elicits DA release in the nAcb by activating nAChRs (Sziraki et al., 1999; Johnson et al., 2000; Seppa and Ahtee, 2000; Gaddnas et al., 2002; Shim et al., 2001; Green et al., 2001; De Villiers et al., 2002; Mogg et al., 2002). The role of these nAChRs in regulating DA release was originally investigated in synaptosome preparations (Rapier et al., 1988; 1990; Grady et al., 1992; Marks et al., 1993; El-Bizri and Clarke, 1994). Nicotine evoked and increased the firing of mesencephalic dopaminergic neurons (Picciotto et al., 1998; Pidoplichko et al., 1997) and stimulated the release of DA from nAcb by acting on β2-containing nAChR (Picciotto et al., 1998). Nicotine elicits a robust increase in DA release from synaptosomes or slices that persists for several seconds or minutes, depending on the concentration of applied nicotine (Rapier et al., 1988, 1990; Rowell, 1995; Sacaan et al., 1995). In addition, the activation of nAChRs at the preterminal location of the axon elicits action potential firing that consequently opens voltage-dependent $Ca^{2+}$ channels in the terminals to enhance transmitter release. Blocking action potentials by TTX removes heterosynaptic modulation of DA release by nicotine agonists (Marshall et al., 1996). Soliakov and

Wonnacott (1996) demonstrated further that voltage-gated $Ca^{2+}$ channels contribute to nAChR-mediated facilitation of DA release.

Microdialysis *in vivo* demonstrated that nicotine can augment DA levels and may be due in part to activation of nAChRs on DA terminals. This is because nicotine-elicited DA release can be seen in purified synaptosome preparations from the nAcb that are highly enriched in presynaptic elements (Grady et al., 1992; Rowell, 1995; Wonnacott et al., 1990). Although injection of a nicotinic agonist directly into the DA terminal fields of the nAcb can stimulate DA release, the strongest effects of nicotine appear to be on the DA cell bodies of the VTA (Mifsud et al., 1989). Nicotine self-administration also results in the increased expression of Fos-related antigens in the nAcb (Merlo-pich et al., 1997), which are transcription factors that may be involved in neuroadaptation following chronic treatment with drugs of abuse (Kelz et al., 1999; Nye and Nestler, 1996).

Nicotine reinforcement has been studied in knock out mice lacking the 22 subunit of the nAChRs (Picciotto et al., 1998). In the absence of the 22 subunit, neurons in the DA system are grossly normal, but nicotine fails to induce DA release in the nAcb, and DA neurons in the VTA become unresponsive to nicotine. Nicotine self-administration is also abolished in these mice, suggesting that the 22 subunit is an important component of the nAChR mediating nicotine reinforcement.

Studies using glutamatergic receptor antagonists along with K7-selective subunit antagonist, MLA in rats have suggested that K7 subunit-containing receptors localized presynaptically on glutamatergic afferents in the VTA contribute to the stimulation of DA release in the nAcb (Schilstrom et al., 1998a,b). It is worth mentioning that even after continuous 50-day administration, nicotine still continues to activate nAChRs regulating

accumbal DA release and mecamylamine reduced the DA output in nicotine-treated mice (Gaddnas et al., 2002). Mecamylamine can fully antagonize the effect of nicotine in a non-competitive way, while the antagonism by MLA and dihydro-β-erythoidine (DHβE) can be reversed for nicotine-induced DA release (Clarke and Reuben, 1996). Blockade of NMDA receptors in the VTA attenuates the enhancing effect of nicotine on extracellular levels of DA in the nAcb. Moreover, pretreatment with MLA in the VTA abolished nicotine-induced DA releasing in the nAcb, indicating a role for α7 nAChRs in this mechanism (Mogg et al., 2002).

On the other hand, the effects of nicotine on evoking DA release have also been demonstrated using different nAChR agonists. Epibatidine, an agonist of nAChR, significantly increased DA output and elevated the concentration of DOPAC, and also tended to elevate that of HVA in the nAcb. (Seppa and Ahtee, 2000). Nornicotine, a tobacco alkaloid and an active nicotine metabolite, can stimulate DA release from nAcb in a nicotinic receptor-mediated manner (Green et al., 2001).

In addition, finding nicotine-induced stimulation of major reward system of the brain involving the activation of NMDA receptors (Shilstrom et al., 1998a, b; Sziraki et al., 1998) indicates the possible involvement of the nAChRs in psychiatric diseases such as schizophrenia. Considering the nicotinic action of clinically used antidepressants, the results also suggest that nAChR activation may be involved in major depression (Salin-Pascual and Drucker-Colin, 1998).

### 6.3. Facilitation of ACh release

Nicotine-induced ACh release has been reported in several areas of the CNS

including interpeduncular nucleus and striatum (Grady et al., 2001) in mice, thalamocortical terminals in prefrontal cortex (Lambe et al., 2003), striatal slices of rats (Sandor et al., 1991a, b) in which nicotine induces release of ACh from LA neurons in a TTX-sensitive manner, suggesting a postsynaptic and /or presynaptic nAChR-driven positive feedback. The increase in both ACh and glutamate release appears to be mediated by α7 subunit-containing nAChR as it can be blocked by α-bungarotoxin and is greatly diminished in the presence of antisense oligonucleotides targeted to the α7 subunit (Picciotto et al., 1998; Pidoplichko et al., 1997).

### 6.4. Pre- but not post-synaptic nicotinic ACh receptors mediates the facilitation

Recording of nAChR channels and nicotine-induced $Ca^{2+}$ influx in intact presynaptic terminals provides strong evidence that direct activation of presynaptic autoreceptors and heteroreceptors, rather than being retrograde mechanisms, actually modulates transmission at sites where nAChRs are expressed on both pre- and postsynaptic structures (Coggan et al., 1997; Gray et al., 1996). These studies, and others demonstrating that nAChR-mediated synaptic facilitation is dependent on pre- but not postsynaptic changes in internal $Ca^{2+}$, indicate that the nicotinic modulation of synaptic transmission is due to direct activation of nAChRs localized on the presynaptic terminals per se. It is important to note that these studies do not address the role of postsynaptic nAChRs in mediating "classical" synaptic transmission.

## 7. Dopaminergic modulation of neuronal excitability in the nucleus accumbens

### 7.1. Dopamine receptor subtypes

DA receptors were originally differentiated into two major subtypes, D1 and D2 (Tarazi et al., 1998). Recent technical advances in molecular genetics led to the discovery of additional DA receptor subtypes (D3, D4, D5). These proteins have generally lower relative abundance and more restricted cerebral localization than the classical D1 and D2 receptors, but many molecular and pharmacological similarities to the original receptor types. Based on their similarities, DA receptors are considered to form D1-like (D1, D5) and D2-like (D2, D3, D4 and their variants) families. D1-like receptors are positively coupled to AC through activating G protein ($Gs/o_{lf}$), and by stimulating AC, elevating cytosolic cAMP. This second messenger, in turn, induces the dissociation of the regulatory subunit from cAMP-dependent protein kinase A (PKA). By phosphorylating targeted proteins, PKA alters a variety of cellular functions. There is also evidence that D1 and D5 receptors can activate other classes of G proteins, such as $G_o$ and $G_z$ proteins (Sidhu, 1998). D2-like receptors are thought to exert an opposite influence on AC activity. These receptors activate $G_i/_o$ proteins which inhibit AC.

### 7.2. Dopaminergic receptor expression in the nucleus accumbens

Five subtypes of DA receptors have been detected in the nAcb. D1 and D3 receptors are more abundant in the nAcb than in the dorsal striatum and are correlated with the expression of SP (Lu et al., 1998; Schwartz et al., 1998; Tarazi et al., 1998). D2 receptor expression appears to be correlated with enkephalin expression in the nAcb. D4 and D5 receptor expression is much lower (Sibley, 1995) and the results from Yan and Surmeier (1997) suggested robust D5 receptor expression by nAcb LA neurons.

### 7.3. Dopaminergic modulation of neuronal activities in the nucleus accumbens

### 7.3.1. Modulation of ionic conductance

Several studies have demonstrated that DA or DA receptor agonists modulate ionic conductance in the nAcb. DA could cause three types of membrane responses specifically. These are hyperpolarization, depolarization and hyperpolarization followed by a depolarization in nAcb neurons of guinea-pig brain slices (Uchimura et al., 1986). Further studies revealed that the DA-induced hyperpolarization is produced by an activation of the D1 receptor and involved an increase in $K^+$ conductance, whereas DA-induced depolarization was generated by the activation of the D2 receptor accompanied by a decrease in $K^+$ conductance (Uchimura et al., 1986). Zhang et al. (2002) recently found that D1 receptor stimulation suppresses N- and P/Q-type $Ca^{2+}$ currents by activating a cAMP/PKA/protein phosphatase signaling system and that repeated cocaine treatment reduces N- and R-type, but not P/Q- or L-type currents. In addition to modulating $K^+$ and $Ca^{2+}$ currents, DA probably also affect $Na^+$ currents. Amphetamine, an indirect DA agonist promoting DA release, inhibits field potentials and $Na^+$ currents in rat nAcb neurons (Huang and Lin, 1998). Also repeated cocaine injections reduced whole-cell $Na^+$ currents in nAcb slice preparations. Freshly dissociated nAcb neurons from cocaine-pretreated rats also exhibited diminished $Na^+$ current density and a depolarizing shift in the voltage-dependence of $Na^+$ channel activation (Zhang et al., 1998). Like in the nAcb, D1 receptors modulating $Na^+$ current has also been found in the dorsal striatal cells (Schiffmann et al., 1995). Consistent with the findings in the nAcb, either DA or SKF38393, a D1 receptor agonist, produced a reversible inhibition of action potential discharge evoked by intracellular depolarizing current pulses. This inhibitory

effect was antagonized by the D1 receptor antagonist in dorsal striatum (Calabresi et al., 1987, 1988).

### 7.3.2. Modulation of synaptic transmission

Several studies have found that DA depressed excitatory synaptic transmission in the nAcb by acting on D1 receptors (Pennartz et al., 1992; Harvey and Lacey, 1996; Nicola et al., 1996; Nicola and Malenka, 1997, 1998). The D1 receptor-mediated reduction of EPSPs/EPSCs observed *in vitro* appears to be a presynaptic effect because the paired-pulse ratio was increased by DA (Pennartz et al., 1992; Nicola et al., 1996). DA also reduced the frequency of spontaneous miniature EPSCs (Nicola et al., 1996). The inhibitory effect of DA does not appear to be dependent on elevated cAMP levels, in spite of a clear enhancement of EPSC by forskolin (Harvey and Lacey, 1996; Nicola and Malenka, 1997). In contrast, several studies *in vitro* in the dorsal striatum have found that DA enhanced NMDAR-synaptic or iontophoretic responses via the activation of D1 receptors (Cepeda et al., 1993, 1998; Levine et al., 1996a, b), whereas other groups failed to find a facilitatory effect of D1-like receptor activation on NMDAR-responses in the dorsal striatum (Calabresi et al., 1995; Nicola and Malenka, 1998). Stimulation of excitatory afferents evokes a dual component EPSP/EPSC mediated by AMPA receptors and NMDA receptors in MS neurons (Chang and Kitai, 1985; Pennartz et al., 1991; Kombian and Malenka, 1994). A provocative suggestion is that DA can have distinct effects on these two components depending on the subtype of DA receptor activated. Specifically, D2 receptor activation has been reported to reduce AMPAR-EPSPs as well as the response to iontophoretically applied glutamate or AMPA (Cepeda et al., 1993; Hsu et al., 1995; Levine et al., 1996b), whereas D1 receptor activation is reported to

enhance synaptic or iontophoretic NMDAR-responses (Cepeda et al., 1993, 1998; Levine et al., 1996a, b). On the other hand, Levine et al. (1996b) found that AMPAR-EPSP was not be altered by D1 receptor activation.

Calabresi and colleagues (1995) have reported no effect of DA and D1 or D2 receptor agonists on the responses of dorsal striatal cells to iontophoresed AMPA or NMDA. Consistent with these observations, no effects of DA on excitatory synaptic responses in field potential or whole-cell recording from dorsal striatal region was found (Malenka and Kocsis, 1988; Nicola and Malenka, 1998), despite the discovery of robust effects of DA on excitatory responses in nAcb region of the striatal-nAcb slice (Nicola and Malenka, 1998).

The inhibitory effect of D1 receptor activation on excitatory transmission appears to be mediated by presynaptic mechanism (Pennartz et al., 1992; Harvey and Lacey, 1996; Nicola et al., 1996; Nicola and Malenka, 1997, 1998) whereas a postsynaptic mechanism is still the subject of some controversy. Based on the findings that adenosine A1 receptor antagonists blocked the effects of DA on EPSCs, and that postsynaptic D1 receptor activation increased synaptic NMDAR-responses, Harvey and Lacey (1997) concluded that the enhanced NMDA current was responsible for the release of adenosine, which presynaptically inhibited glutamate release. This hypothesis is consistent with the anatomical localization of D1 receptors on postsynaptic dendritic spines and shafts of nAcb cells (Huang et al., 1992; Yung et al., 1995; Caillé et al., 1996). However, DA enhancement of NMDAR-EPSCs could not be replicated (Nicola and Malenka, 1997; Zhang and Warren, 1998). In experiments with isolated EPSCs, we found that DA (50 μM) strongly inhibits NMDAR-EPSCs and moderately inhibits AMPAR-EPSCs in the

nAcb slices from young rats (Zhang & Warren in preparation, see chapter IV of present thesis).

**8. Aims of the present study**

The present study was performed in order to examine the characteristics of nAcb EPSCs and their modulation by ACh and DA during postnatal development in rats. The experiments were carried out in MS neurons of the nAcb slices using whole-cell patch-clamp recording technique in postnatal rat (P0-P71). The feature of postnatal development of EPSCs was selected as a terminal point in these experiments. The present thesis is composed of three articles, which have been published or will be submitted to journals for publication and presented in Chapter II, III, and IV, respectively.

As reviewed above, glutamatergic receptors and their mediated EPSCs have been suggested to be one of the most important excitatory drive in the nAcb, whereas ACh and DA appear to be two important neurotransmitters in modulating nAcb function. Previous study has shown that the physiological properties of MS neurons during postnatal development become adult-like only by the end of the third postnatal week, suggesting that nAcb function is different during early postnatal period from that in adults (Belleau and Warren, 2000). Presumably, there is a critical period of synapses formation and consolidation during which the transitory deprivation of some of nAcb afferents will produce an imbalance between the strength of different inputs and lead to a pathological state.

Thus, it would be important, first to determine whether there is any change in glutamatergic receptors-mediated EPSCs during early postnatal development, and to characterize the features of NMDAR- and AMPAR-EPSCs during this period. The second part of the present thesis investigated the cholinergic modulation of EPSCs during postnatal development using different cholinergic receptor agonists and antagonists. In the third part of this thesis, we studied the modulatory role of the dopaminergic systerm on EPSCs during early synaptic development. We found that NMDAR-EPSCs are the largest during the second postnatal week and decline during the following weeks, suggesting that NMDA receptor may play a critical role during synaptic maturation during postnatal development. DA preferentially inhibited NMDAR-EPSCs compared to AMPAR-EPSCs. These effects were mediated by D1 type receptor mainly through both pre and postsynaptic mechanisms. Activation of the cholinergic receptors produced a dual modulatory function, e.g. an excitatory nicotinic action and an inhibitory muscarinic effect on EPSCs during postnatal development. Our results reveal predominant expressions of NMDAR-EPSCs and the contrasting effects of M1 receptor and nAChR on EPSCs as well as the inhibitory effect of activating D1 receptors on NMDAR-EPSCs during postnatal development. Presynaptic mechanism mediates the actions of ACh. Pre and postsynaptic mechanisms may be involved in the effects of DA on excitatory synaptic transmission. Predominant expression of NMDA receptors and hyperfunction of the dopaminergic system during early postnatal development might play a pivotal role in the etiology of the schizophrenia (Joyce and Meador-Woodruff, 1997).

# CHAPTER II

**In preparation**

# POSTNATAL DEVELOPMENT OF EXCITATORY POSTSYNAPTIC CURRENTS IN NUCLEUS ACCUMBENS

Liming Zhang[1,2] and Richard A. Warren[1,3]

[1]Centre de recherche Fernand-Seguin, [2]Department of Physiology and [3]Department of Psychiatry

University of Montréal, Montréal, Canada

***Running title:*** DEVELOPMENT OF EPSCS IN NUCLEUS ACCUMBENS

***Address for correspondence:***

Richard A. Warren
Centre de recherche Fernand -Seguin
7331 Hochelaga street
Montréal, Québec, Canada, H1N 3V2
Tel. (514) 251-4015 ext. 3415
Fax (514) 251-2617
[redacted]

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**ABSTRACT**

We recorded excitatory postsynaptic currents (EPSCs) evoked by local electrical stimulation in 243 nucleus accumbens (nAcb) neurons *in vitro* during postnatal development from the day of birth (postnatal day 0; P0) to P27 and in young adults rats (P59-P71). An EPSC sensitive to glutamatergic antagonists was found in all neurons. In the majority of neurons (189/243), the EPSC had two distinct components: an early one sensitive to 6-cyano-7- nitroquinoxaline -2,3-dione (CNQX) and a late one that was sensitive to d-2-amino-5- phosphonovaleric acid (APV) showing that early and late components of the EPSC were mediated by AMPA/KA and NMDA receptors respectively. During the first few days after birth, the amplitude of both the early and late components of the EPSC were relatively small and then began to increase until the end of the second postnatal week. Whereas the characteristics of the early component appeared to stabilize from that point on, the late component began to decrease in amplitude and became virtually absent in preparations from more than 3-week-old animals. In addition, the ratio of the late to early component amplitudes of the EPSCs followed a developmental pattern parallel to that of the late component showing an increase during the first two postnatal weeks followed by a decrease. Together, these results show the presence of transiently predominant expression of NMDA receptor-mediated EPSCs over AMPA/KA receptor-mediated EPSCs during the first 3 postnatal weeks. The time frame could represent a critical developmental period of the nAcb.

**Key Words:** Nucleus accumbens; development; excitatory postsynaptic currents; NMDA receptor; AMPA/KA receptors; plasticity; schizophrenia.

## RÉSUMÉ

Nous avons enregistré les courants postsynaptiques excitateurs (CPSEs) évoqués par une stimulation électrique locale dans 243 neurones du noyau accumbens (nAcb) *in vitro* pendant le développement postnatal à compter du jour de la naissance (jour postnatal 0; P0) jusqu'à P27 ainsi que chez de jeunes rats adultes (P59-P71). Un CPSE sensible aux antagonistes glutamatergiques a été identifié dans tous les neurones. Dans la majorité des neurones (189/243), le CPSE comprenaient deux composantes distinctes: une précoce sensible au *6-cyano-7-nitroquinoxaline-2,3-dione* (CNQX) et une tardive sensible à la *d-2-amino-5-phosphonovaleric acid* (APV) démontrant qu'ils étaient respectivement médiés par des récepteurs de type AMPA/KA et NMDA. Pendant les premiers jours après la naissance, l'amplitude des deux composantes des CPSEs étaient relativement petite et ont ensuite augmenté jusqu'à la fin de la deuxième semaine postnatale. Alors que les caractéristiques de la composante précoce se sont stabilisées à partir de cet âge, l'amplitude et la durée de la composante tardive ont commencé à diminuer et elle est devenue difficile à évoquer dans des préparations d'animaux âgés de plus de 3 semaines. En outre, le rapport entre l'amplitude de la composant tardive et de la composante précoce du CPSE a suivi un modèle développemental semblable à celui de la composante tardive montrant une augmentation pendant les deux premières semaines postnatales suivie d'une diminution. Ensemble, ces résultats montrent la présence d'une expression prédominante des CPSE médiés par des récepteurs NMDA pendant les 3 premières semaines postnatales. Cette période pourrait représenter une période critique dans le développement postnatal du nAcb.

**Mots-Clés :** Noyau accumbens; développement; courants postsynaptiques excitateurs; récepteur NMDA; récepteurs AMPA/KA; plasticité; schizophrénie.

## INTRODUCTION

Developmental plasticity in the central nervous system has been well documented in the visual and somatosensory systems where there is a critical period during which typical development depends upon normal sensory input from the environment (Wiesel and Hubel, 1963; Rhoades et al., 1990). The absence of appropriate peripheral input during that period leads to irreversible alteration of perception. Presumably, similar processes operate throughout the neuraxis during postnatal development but they have been seldom documented outside sensory systems. Just as the lack of normal inputs in sensory systems during the critical period leads to altered perception, the lack of normal inputs in limbic structures could lead to mental disorders and constitute the organic basis of some psychiatric illnesses that may involve neurodevelopmental processes (Raedler et al., 1998).

The nucleus accumbens (nAcb) is a part of the limbic system believed to be a center for the integration of limbic and motor systems. It is a major target of the subiculum and the prefrontal cortex, as well as other limbic structures including the entorhinal cortex, the amygdala and midline thalamic nuclei (Kelley et al., 1982; Groenewegen et al., 1987; Phillipson and Griffiths, 1985; Groenewegen et al., 1982; Krayniak et al., 1981; Groenewegen et al., 1980; Newman and Winans, 1980; Kelley and Domesick, 1982; Jayaraman, 1985). These afferents are thought to be primarily glutamatergic and some of them including those from prefrontal cortex, hippocampal formation and amygdala converge on single nAcb projection neurons, the medium spiny (MS) neurons (O'Donnell and Grace, 1995). Interactions between these different inputs, combined with the intrinsic properties of MS neurons (Wickens and Wilson, 1998;

Wilson and Kawaguchi, 1996) result in the expression of two stable membrane potential states in these neurons: a hyperpolarized state during which MS neurons are silent, and a depolarized state during which they are active (O'Donnell and Grace, 1995). To achieve this high degree of functional integration, the glutamatergic innervation of the nAcb has to be finely tuned during development. Presumably, a transitory deprivation of some of nAcb afferents during the critical period of synapses formation and consolidation will produce a long lasting imbalance between different inputs and lead to pathological states. In the present study, we have examined the excitatory postsynaptic currents (EPSCs) during early postnatal development and found that the NMDA receptor-mediated EPSCs are expressed transiently in nAcb neurons, reaching their maximum toward the end of the second postnatal week to become virtually absent by the end of the third postnatal week. We suggest that the time during which NMDA receptor-mediated EPSCs are present constitute a critical period during which the disturbance of nAcb inputs could lead to permanent loss of function. Parts of the present study have been published in abstract form (Zhang et al., 1998; Zhang and Warren, 1999).

## METHODS

***Slice preparation.*** Detailed procedures appear elsewhere (Belleau and Warren, 2000). Briefly, 400μm parasagittal slices containing the nAcb were obtained from the day of birth (P0) up to P71 rats. Slices were incubated for at least one hour before recording was started in a submerged type chamber superfused with room temperature artificial cerebrospinal fluid (ACSF) containing (in mM) 126 NaCl, 26 $NaHCO_3$, 10 dextrose, 3 KCl, 1.3 $MgSO_4$, 2.5 $CaCl_2$, 1.25 $NaH_2PO_4$ with a pH of 7.4 when bubbled with a gas mixture of 95% $O_2$ and 5% $CO_2$. The nAcb was visualized with a stereo microscope using the anterior commissure, the neostriatum, the septum and the ventricles as landmarks based on Paxinos and Watson (1986).

***Recording and electrical stimulation.*** Whole-cell recording was achieved using the blind patch-clamp technique (Blanton et al., 1989) with an Axoclamp-2B amplifier (Axon Instruments) in single electrode voltage clamp mode. Recording pipettes had a resistance between 3 and 6 MΩ when filled with a solution containing (in mM) 135 CsFl, 2 $MgCl_2$ , 1 $CaCl_2$, 11 EGTA, 10 HEPES and 2 $K_2$-ATP with a pH of 7.3 ±0.05. QX314 (5 mM; Alomone Laboratories) and 0.3% neurobiotin were routinely added to the recording solution to block voltage-dependent $Na^+$ channels and to label recorded neurons. The potential reference on the Axoclamp-2B was adjusted to zero prior to achieving whole-cell configuration. Data acquisition was realized using pClamp 6.0 software (Axon Instruments). Local electrical stimulation was accomplished by using a monopolar tungsten microelectrode placed at least 2mm away from the recording electrode either within nAcb or just outside its border. In order to adjust the stimulus

strength, the voltage was gradually increased until the postsynaptic response at a holding membrane potential of -70mV reached its maximum. The stimulus was then decreased to get a stable response that was around 90% of the maximum. With the membrane potential holding at -70mV, the synaptic response was recorded at membrane potentials between -100 to +40mV in 20mV increments using 350ms voltage steps that were administered every 8-20s depending on the age of the preparation. A single anodal electrical stimulus (0.1ms, 3-7V) was superimposed at 150ms into the voltage step. Four to eight traces were recorded at each membrane potential ($V_m$) and averaged offline. Pharmacological agents used to isolate EPSCs including bicuculline methiodine (BMI), d-2-amino-5-phosphonovaleric acid (APV) and 6-cyano-7- nitroquinoxaline-2,3-dione (CNQX) (all obtained from Tocris) were added to the superfusing medium to final concentrations of 10, 50 and 20μM, respectively.

***Analysis.*** Data analysis was done utilizing Cambridge Electronic Design software. The amplitude of the evoked synaptic current ($I_R$) was plotted as a function of $V_m$ at two different time points: one at an early point corresponding to the peak of the inward current recorded at -100mV, and a later one at a point when the fast inward current recorded at -100mV had decayed into a slow depolarizing plateau almost parallel to the baseline (e.g. Fig. 1). At the time the later component of the response was measured, the early component had decayed by an average of 89%. The offset potential, measured upon electrode withdrawal from the cell, was accounted for by assuming that it drifted in a linear fashion with time from the start of the recording session. All membrane potential values have been corrected for liquid junction potentials of -14mV (Barry, 1994). Throughout the text, membrane potentials are reported to the closest 10mV whereas exact

membrane potentials have been used for calculations. Statistical analysis was performed using SigmaStat (SPSS Inc.) and, when necessary, raw data were logarithmically transformed to fulfill the requirements of parametric statistical tests. Non-parametric statistical tests were used only if this procedure failed (Sokal and Rohlf, 1995). Results are presented as mean ± standard error.

***Morphology.*** Following electrophysiological experiments, slices were placed in a fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 for 24 hours. Some slices were cryoprotected in a 30% glucose solution and re-sectioned 60μm thick on a freezing microtome whereas most slices were whole processed for neurobiotin using standard procedures. Sections and slices containing labeled cells were imaged using a CCD camera (Dage model DC330) mounted on a microscope (Nikon model Optiphot 2) using 4x, 10x and 40x objectives and the images were stored on a computer using Image Pro Express software (Media Cybernetics). Cell body measurements were made using Image Pro Express software. Final images montages were assembled using Corel Photo-Paint software using images taken with a 10x objective and printed using a dye sublimation printer (Sony model UP-5600MD). Neurons filled during other series of experiments on the development of the nAcb (Zhang and Warren, 1999, 2002) have been included in the present analysis.

## RESULTS

Postsynaptic currents evoked by local electrical stimuli of nAcb were recorded in over 243 nAcb neurons in slices from animals between P0 and P71. In a subset of 41 neurons recorded in preparations from comparable ages (between P1 and P71) in normal ACSF, the addition of BMI (10μM) produced a 46 ± 3mV positive shift in the reversal potential of evoked postsynaptic currents from -38 ± 4mV to 8.4 ±3.7mV, suggesting that a significant $GABA_A$-mediated component was present early after birth and throughout the postnatal period examined. With BMI present in the bathing solution, postsynaptic currents were completely abolished when the glutamatergic receptor antagonists CNQX (20μM) and APV (50μM) were added to the superfusing medium (e.g. Fig.1B) suggesting that under these conditions postsynaptic currents consisted of isolated ionotropic glutamatergic EPSCs. Following these initial experiments, BMI (10μM) was routinely added to the ACSF in order to isolate and record glutamatergic excitatory postsynaptic currents (EPSCs). $I_R$-$V_m$ relationships of EPSCs were obtained from recording 243 nAcb neurons in 84 experiments using nAcb slices obtained from P0-P27 (231 cells) and P59-P71 (12 cells) animals.

### *Characteristics and nature of the EPSCs*

EPSCs could be readily evoked in preparations of the nAcb from P0 animals and they were found in all recorded neurons, although their characteristics changed substantially during the postnatal period. The most commonly encountered type of response consisted of an early, fast, inward current having a linear relationship with the membrane potential, followed by a late and extended inward current having a non-linear relationship with membrane potential. An example of postsynaptic currents recorded

between holding membrane potentials of -100 and +40mV with corresponding $I_R$-$V_m$ curves from a representative neuron is shown in Figure 1A, left and right panels respectively. In this case, an inward current with a peak occurs 13 ms after the stimulus rapidly developed at membrane potentials -40mV and below (Fig. 1A, *Early* labeled vertical dotted line). This early, inward current decayed with a similar time course at membrane potentials between -100 and -60mV (3 lower traces) whereas at -40mV the decay of the response was altered by a second inward current having a much slower time course (Fig. 1A, *Late* labeled dotted line). The $I_R$-$V_m$ curves (Fig. 1A, lower panel) showed that the early current had a close to linear relationship with the membrane potential ($r = 0.986$ in this case), whereas the late current was virtually absent at negative membrane potentials and became apparent only at membrane potentials -40mV and above. The early postsynaptic current had a constant peak latency and appeared to decay at the same rate at membrane potentials below -40mV, suggesting that late inward current contamination was minimal at these membrane potentials (e.g. Fig. 1).

Figure 1.

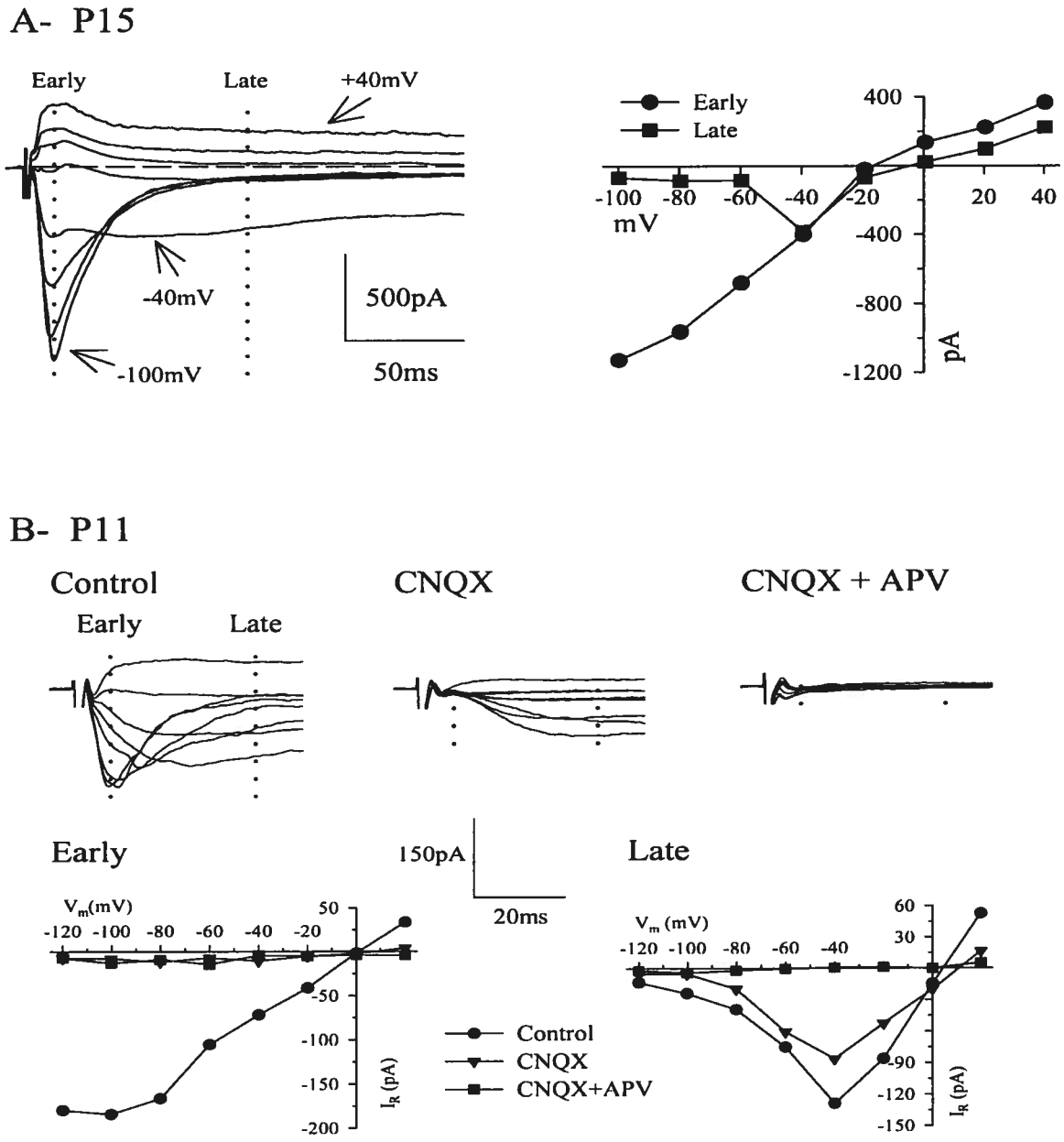


**Figure 1. A.** Postsynaptic currents recorded in a nAcb neuron from a P15 slice preparation (left) and corresponding $I_R$-$V_m$ curves measured 13ms (early) and 106ms (late) after the stimulus (right). The early inward current at -100mV peaked 13ms after the stimulation, whereas the late response was measured 93ms later, when the early inward current had almost completely decayed. The $I_R$-$V_m$ relationship of the early inward current was linear ($r = 0.986$, $a = 11.429$, $b = 30.56$), whereas the late component was voltage-dependent with a peak around -40mV in the negative membrane potential range. **B.** Effects of ionotropic glutamatergic antagonists on the EPSCs recorded in a neuron from a P11 preparation. The upper row shows the response traces recorded between -120mV and +20mV in incremental steps of 20mV before (Control), during the bath application of CNQX (20μM; CNQX) and CNQX and APV (50μM; CNQX + APV). The lower row shows the $I_R$-$V_m$ relationships measured at the time point labeled Early (8ms after stimulus onset) and Late (40ms after stimulus onset) in the first panel of the upper row before and during the application of glutamatergic antagonists.

The characteristics of the early component of the EPSCs were measured at the membrane potential of -100mV in order to minimize possible contamination by the late component as the latter appeared virtually absent at this membrane potential. At –100 mV, the early postsynaptic current peaked 4-21ms (9.5 ± 0.2ms, n=243) after electrical stimulus onset and decayed to a small steady state plateau in 13-126ms (40 ±1ms, n=243). The late component of the response was measured after the early component of the EPSCs had decayed, 20-138ms (49 ±1ms, n=243) after stimulus onset in order to reduce contamination by the early component of the EPSC as much as possible. Under these conditions, a late EPSC with a non-linear V-shaped current-voltage relationship, typical of currents mediated by NMDA receptors, was found in 189 neurons. In the remaining 54 cells, no non-linear current-voltage relationship could be found at these or longer latencies. No statistically significant difference was found between neurons displaying a late EPSC and neurons lacking one for the peak latency of the early response (9.3 ± 0.2ms vs 10.2 ± 0.4ms respectively; $T_s$ = 7280.5, p = 0.129), its decay time (39 ± 1ms vs 41 ±3ms respectively; $t_s$= 0.0697, df= 241, p = 0.944) and its amplitude (269 ± 14pA vs 221 ± 18pA respectively; $t_s$ = 2.013, df = 241, p = 0.052), suggesting that the early response was similar in neurons with and without a late response.

The peak amplitude of the early component of the EPSC was measured at a membrane potential of -100mV and that of the late current at the membrane potential at which it appeared to be in the negative range of membrane potentials (usually –40mV or -20mV, e.g. Fig. 1A) and after the early response had decayed. With these measuring parameters, the early component of the response was on average larger than the late component, being -265 ±14pA (n= 243) vs -125 ±8pA (n=189) respectively ($t_s$ = 12.077,

df= 430, $p < 0.001$).

Overall, the characteristics of the EPSCs strongly suggested that early and late components of the EPSC represented glutamatergic AMPA/KA and NMDA receptors-mediated responses respectively. These assumptions were tested by adding the specific glutamatergic antagonists CNQX and APV to the superfusing medium. A representative example recorded in a neuron from a P11 preparation substantiating this hypothesis is shown in Figure 1. During the control period preceding the administration of an antagonist (Fig. 1B, ***Control*** labeled panel), the EPSC characteristically comprised an early and a late component. The addition of the AMPA/KA receptor antagonist CNQX to the superfusing medium completely abolished the early component of the response at all membrane potentials, while producing only marginal effects on the late component (Fig. 1B, ***CNQX*** labeled panel). Further addition of APV, abolished the late response leaving no detectable postsynaptic current (Fig. 1B, ***CNQX*** + ***APV*** labeled panel). The $I_R$-$V_m$ relationships of the early and late component before and during antagonists superfusion are shown in the bottom left and right panels of Figure 1B respectively.

CNQX was tested in 33 neurons and produced a decrease of the early component of the response averaging 91 ± 2% (ranging from 46 to 100%) whereas APV produced an average decrease of the late component of 85 ± 5% (ranging from 44 to 100%) when tested in 14 neurons displaying a late component. From these results we concluded that the early EPSC was mainly mediated through activation of AMPA/KA receptors, whereas the late component was mediated by the activation of NMDA receptors.

Although the largest effects of CNQX and APV were on the early and late components of response respectively, there were some significant exceptions to the

pattern described above, especially in preparations from very young animals. In some cells, CNQX and APV non-selectively antagonized both the early and late components of the response. Figure 2A illustrates such a case in which APV reversibly decreased both the late and early components of the EPSC in a neuron recorded from a P2 animal preparation. During the control period (Fig. 2A, ***Control*** labeled panel) both an early and late component of the EPSC was apparent in the current traces, as well as in the corresponding $I_R$ -$V_m$ curves. When APV was added to the superfusing medium, both the early and late components of the EPSC were completely abolished (Fig. 2A, ***APV*** labeled panel) and these effects were reversible (Fig. 2A, ***Wash*** labeled panel). APV produced similar effects, within the range of those produced by CNQX on the early component of the EPSC (74 ± 7%; ranging from 45 to 98%) in 9 of 21 neurons with an early component that were tested; 7 of these neurons were recorded in preparations from animals less than a week old. These results suggest that in some neurons, the EPSC was mediated only by NMDA receptors.

Figure 2.

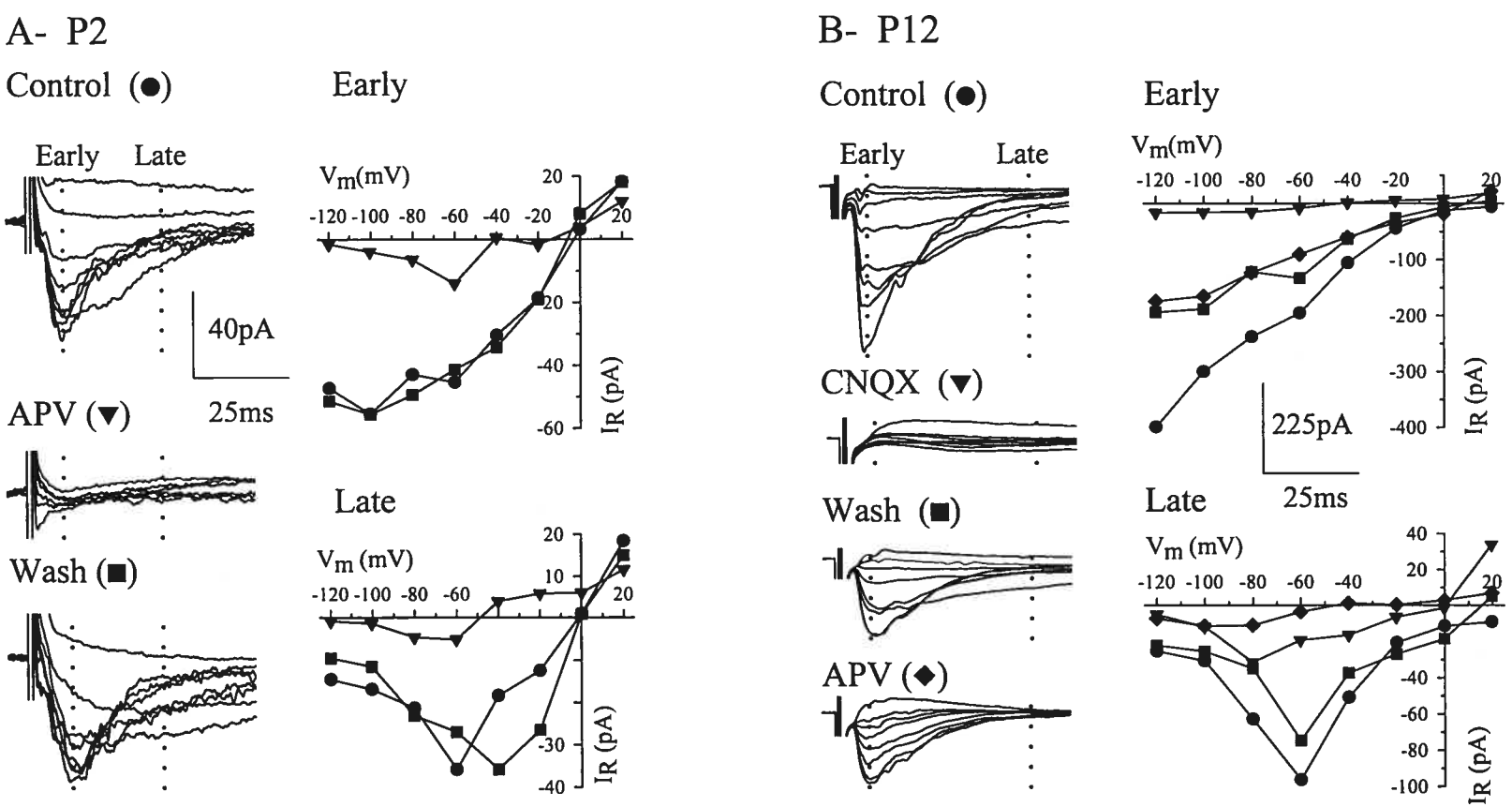


**Figure 2.** Effects of ionotropic glutamatergic antagonists on the EPSCs of nAcb neurons from a P2 (**A**) and a P12 (**B**) animal. In **A**, the left panel shows the current traces before (Control,●), during (APV, ▼) and after (Wash, ■). The addition of APV (50 μM) to the superfusing ACSF with the corresponding $I_R$-$V_m$ curves was shown in the right panel for the early and late component of the EPSCs. Conventions are similar in **B** except that CNQX (20μM, ▼) was used and APV (50 μM,◆) was also tested after CNQX was washed out (■). The measurements of the early and late responses were made at 10 and 36ms, respectively after the stimulus onset in **A** and 9 and 50ms in **B**. Other labels and conventions in **B** are similar to **A**.

In some neurons, CNQX appeared to abolish both the early and late components of the EPSCs. It produced a significant reduction (69 ± 7%; ranging from 54 to 97%) of the late response in 5 of 17 neurons tested. An example is shown in Figure 2B. During the control period, the EPSC comprised an early and a late component, which were both abolished when CNQX was added to the superfusing medium. Following wash out of CNQX, the addition of APV antagonized only the late component whereas the early component appeared unaffected. These results show that in some cases CNQX could reduce significantly the late NMDA receptor-mediated component of the EPSCs.

***Developmental characteristics of postsynaptic currents***

In neurons in which the EPSC consisted of both early and late components, the ratio between the two components appear to vary significantly and this was related to the age of the preparation. To illustrate that point, we have characterized the EPSCs according to the relative apparent magnitude of the late component of the response.

***Type I response***

In a subset of 25 neurons (10% of the sample), the peak inward current was not observed when the membrane potential was held at -100mV but was seen at a more depolarized membrane potential of -60 or -40mV. Concurrently with the increase in peak current, the response became broader, and the latency to peak often increased by several milliseconds between -100mV and more depolarized membrane potentials. This kind of response was named Type I.

Figure 3A shows a representative Type I response recorded from a P3 neuron. In the current traces of the response (Fig. 3A upper panel), only an early fast inward current was evoked by local electrical stimulation at a holding membrane potential of -100mV.

At -60mV, this response reached its maximum, increasing by more than 40% as compared to the response obtained at -100mV. There was no change in peak latency of the early response between -100 and -60mV although the response appeared broader because of a slower decay rate. In contrast, at -40mV, the peak of the response was delayed by several milliseconds as compared to more negative membrane potentials because of a much slower rise time. The decay rate of the response was also much slower at -40mV and overall. The response was much broader due to the full development of the late component. The lower panel of Figure 3A shows a plot of the $I_R$-$V_m$ relationship of the responses shown in the upper panel. It is apparent that there was a marginal late component in the response below -40mV, whereas between -40 and +40mV the $I_R$-$V_m$ of the late response was linear and reversed around 0mV. The early response $I_R$-$V_m$ curve was linear and parallel to that of the late one between -60 and +40mV where it appeared to saturate and reach a plateau at more hyperpolarized potentials. Similar observations were made in 25 neurons and the increase in the peak EPSC between -100mV and more depolarized membrane potentials ranged from 9 to 322pA with an average of 82 ± 16pA (n = 24). In addition, as the membrane was depolarized, the response peak was delayed in 23 neurons by an average of 19 ± 6ms.

In terms of amplitude and time course, Type I responses had on average the smallest early component with the longest time to peak and decay time of all 4 types of responses (Table I). In contrast, they displayed the largest late component amplitude and this even if it was measured 10-15ms later than in Type II and III responses on average (see below). Consequently, the ratio between late and early components was the highest at 0.85 in Type I responses.

Figure 3.

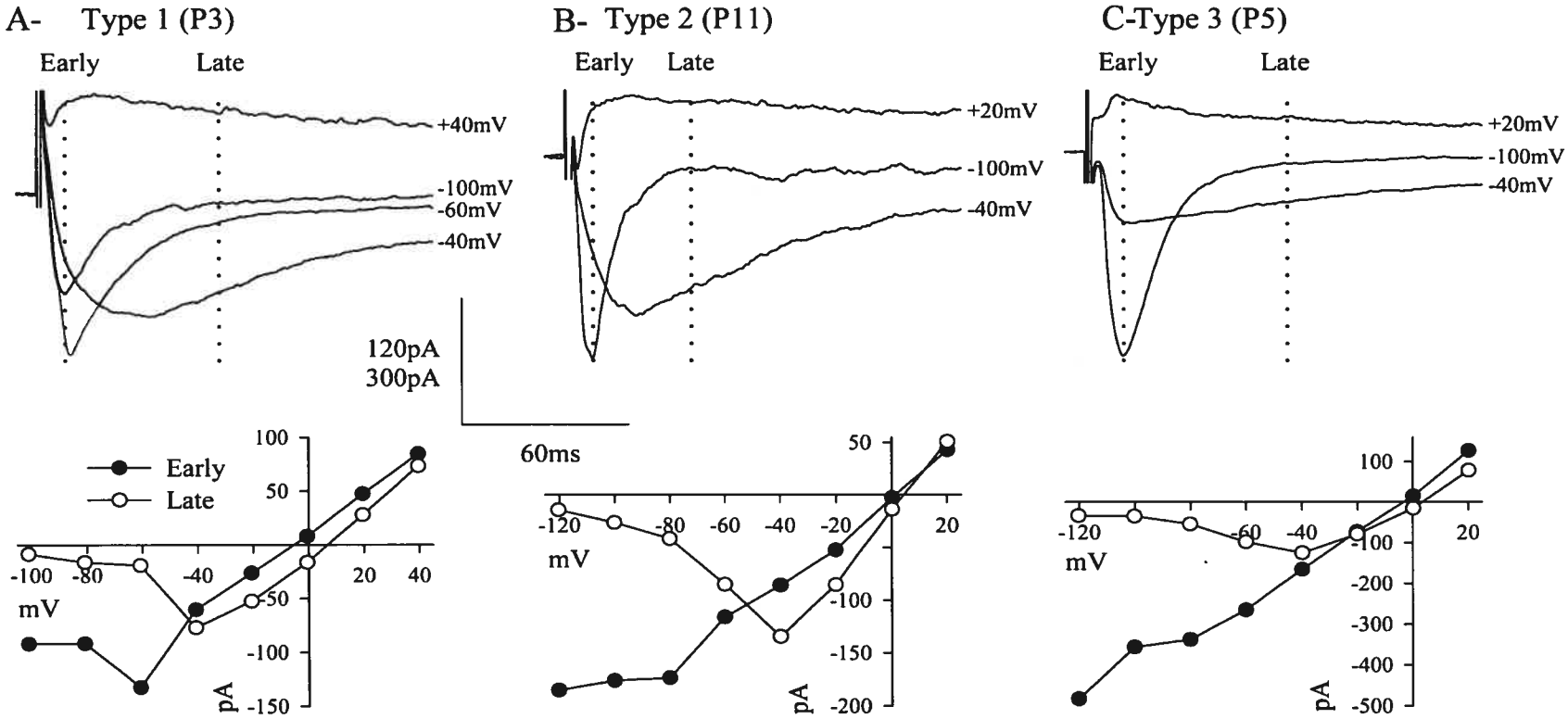


**Figure 3.** Classification of neurons according to the relative size of the late response. **A.** Type 1 responses were characterized by an increase in the peak response as the membrane potential was depolarized from -100mV to -60mV and -40mV. Postsynaptic current traces were recorded in a neuron of the P3 preparation. Each trace represents the average of 6 sweeps. The numbers on the right indicate the membrane potentials to the closest 10mV at which the currents were recorded. Lower panel represents the $I_R$-$V_m$ relationships measured at the dotted vertical lines labelled early and late in the upper panel. **B.** Type 2 responses were characterized by a displacement of the peak to the right as the membrane potential was depolarized from -100mV to -40mV. Postsynaptic current traces were recorded in a neuron of the P11 preparation. Other conventions are as in A. **C.** An example of a Type 3 response in which the late component of the response was in continuity with the early component at -40mV. Postsynaptic current traces were recorded in a neuron of the P5 preparation. Other conventions are as in A. The vertical scale bar represents 120pA for A and B and 300pA for C. The horizontal scale bar represents 60ms for A-C.

**Table 1.** Characteristics of different types of responses

| | Type I | Type II | Type III | No late response | All |
|---|---|---|---|---|---|
| **Early response amplitude (pA)*** | -208 ± 30 (-37 to -729) | -285 ± 21 (-80 to -1126) | -273 ± 23 (-31 to -1208) | -221 ± 18 (-40 to -572) | -258 ± 12 (-31 to -1208) |
| **Late response amplitude (pA)**** | -177 ± 29 (-34 to -733) | -152 ± 12 (-37 to -680) | -86 ± 9 (-13 to -415) | ----- | -125 ± 8 (-13 to - 415) |
| **Early response time to peak at -100mV (ms)***** | 11.1 ± 0.7 (6.6 to 18.1) | 9.4 ± 0.3 (5.6 to 18) | 8.7 ± 0.3 (3.6 to 21) | 10.2 ± 0.4 (5.7 to 20) | 9.5 ±0.2 (3.6 to 21) |
| **Early response decay at -100mV (ms)****** | 49 ± 4 (25 to 104) | 40 ± 2 (15 to 126) | 36 ± 1 (13 to 72) | 41 ± 3 (16 to 107) | 40 ±1 (13 to 126) |
| **Late response latency (ms)******* | 60 ± 4 (32 to 112) | 49 ± 2 (22 to 138) | 45 ± 1 (2- to 93) | ----- | 49 ±1 (20 to 138) |
| **Age (days)** | 8.4 ± 0.8 (P1 to P20) | 9.1 ± 0.4 (P1 to P20) | 8.5 ± 0.6 (P0 to P27) | 21.2 ± 3.2 (P0 to P71) | 11.8 ±0.9 (P0 to P71) |
| **n** | 25 | 77 [1] | 85 [2] | 54 | 242 (189) [2, 3] |

Student-Newman-Keuls method was applied here to test significant variance statistically.

* Significant variance among Type I, II, III and No late responses: $F_s = 3.483$, df = 3, p = 0.017); no pair-wise significant difference was found. Significant variance among Type I, II and III responses ($F_s = 3.372$, df = 2, p = 0.036); multiple test comparison showed significant difference ($p<0.05$) between Type I and Type II. No significant difference for early response amplitude between neurons with a late response and those lacking one: -269 ±14 vs -221±18pA; $t_s = 2.013$, df = 241, p= 0.052.
** Significant variance among Type I, II and III responses: $F_s = 26.799$, df =2, $p<0.001$; Multiple test comparison showed significant difference ($p <0.05$) between Type I and Type III; Type II and Type III.
*** Significant variance among Type I, II, III and No late responses: $F_s = 6.218$, df= 3, $p<0.001$. Multiple test comparison showed significant difference ($p<0.05$) between Type I and Type II; Type I and Type III; Type III and No late response.
**** Significant variance among Type I, II, III and No late responses: $F_s = 3.652$, df= 3, p= 0.013. Multiple test comparison showed significant difference ($p<0.05$) between Type I and Type II; Type I and Type III; Type I and No late response.
***** Significant variance among Type I, II, III and No late responses: $F_s = 7.721$, df= 2, $p< 0.001$. Multiple test comparison showed significant difference ($p<0.05$) between Type I and Type II; Type I and Type III.

[1] One neuron from an adult animal was excluded from averages. [2] One early response value of -2002pA tested as an outlier using Grubbs test ($p<0.005$) and was excluded from the averages. [3] n = 242 for early response characteristics and n = 189 for late response characteristics.

From a developmental point of view, Type I responses were under represented until P3 and were most often encountered between P3 and P13. They were rarely encountered in preparations from more than 2- week-old animals (Fig. 4A).

**Figure 4.**

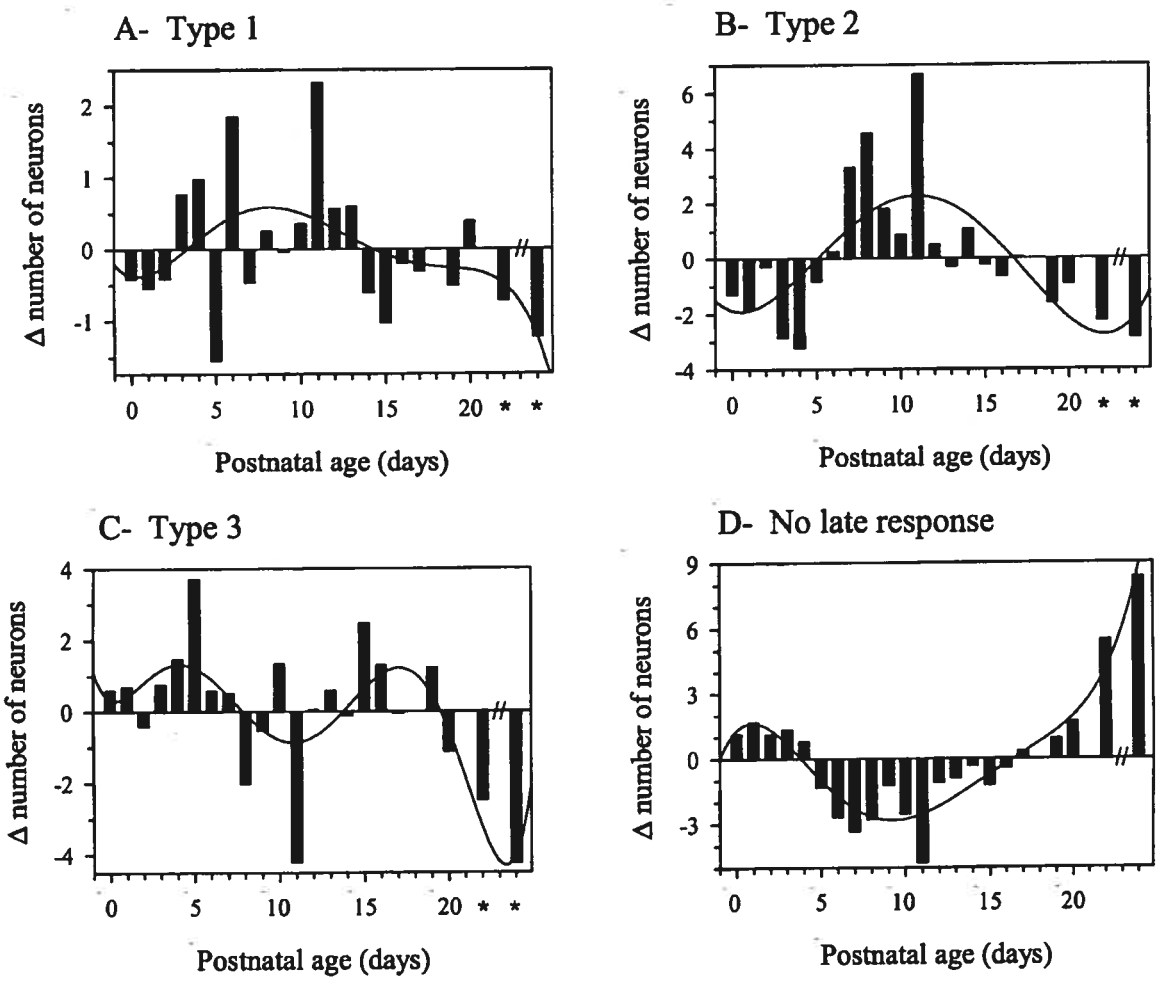


**Figure 4.** Distribution of Type I (A), Type II (B), Type III (C) and no late response (D) neurons as a function of postnatal age. Each graph represents the difference between the number of neurons of each type sampled on a given postnatal day and the number of neurons expected if it was identical to the daily distribution of the whole sample. Upward and downward bins indicate that respectively more and less neurons of this type were sampled than expected on this specific postnatal day. The curves are polynomial regressions to underscore trends in the data. The last two bins of each graph (marked by an asterisk) represent neurons sampled on P21-27 and P59-71 respectively. Note that no neurons were sampled on P18. Number of neurons were 25, 78, 86 and 54 for A, B, C and D, respectively.

***Type II response***

In a second group of neurons (n = 78; 32% of sample), the largest inward current was recorded at the most hyperpolarized membrane potential. However, as the membrane was depolarized, the rise time of the EPSC noticeably increased, delaying the peak of the response by several milliseconds when the late component of the response reached its maximum around -40mV (Fig. 3B, upper panel). The average shift in peak response between holding membrane potentials of -100 and -40mV was 20 ± 1ms (n = 78) and appeared to be independent of the age of the animal used for slice preparation. The $I_R$-$V_m$ relationships of Type II responses resembled that of Type I responses. As with Type I responses, the $I_R$-$V_m$ curve of the early component apparently saturated at hyperpolarized membrane potentials, whereas at depolarized membrane potentials, it was parallel to the non-voltage dependent portion of the late component $I_R$-$V_m$ curve (Fig. 3B, lower panel).

Type II responses were characterized by an early component 37% larger on average than Type I responses, whereas the late component of the EPSCs was not significantly smaller than that of Type I resulting in an average late to early ratio of 0.53 (Table I). In terms of postnatal ages, Type II responses appeared to be under represented between P0 and P6 but were the most commonly found in preparations from animals between P7 and P14 (Fig. 4B).

***Type III response***

In 86 neurons (35% of the sample), the late response was relatively small and was apparent only as a continuation of the early response at membrane potentials above -60mV (Fig. 3C, upper panel). In contrast with Type I and II responses, the rise time of

the early peak response was constant at all membrane potentials. Consequently, no delay in the peak response was observed between hyperpolarized and depolarized membrane potentials. Despite the small size of the late response, its $I_R$-$V_m$ curve was clearly V-shaped with a peak usually around -40mV (Fig. 3C, lower panel). The early component $I_R$-$V_m$ curve was close to a single straight line passing through the origin.

The amplitude of the late component of Type III responses was about half that of Type I and II responses, while the amplitude of the early component was comparable to that of Type II neurons (Table I), resulting in a comparatively small late to early component ratio of 0.32. This response type was also characterized by the shortest early response time to peak and decay time.

Type III responses were most often recorded during the first and third postnatal week, whereas they were comparatively less common during the second postnatal week. As with all neurons with a late EPSC, they were rarely recorded in preparations from animals older than 3 weeks (Fig. 4C).

***No late response***

In 54 neurons (22% of the sample) no late EPSC could be positively identified (not shown). In these neurons, no apparent late response was present, and the $I_R$-$V_m$ curve measured after the decay of the response at -100mV was close to parallel to the abscissa. The AMPA/KA receptor antagonist CNQX was tested in 11 neurons of this type (P1-P71) and produced a large reduction of the EPSC at all membrane potentials with an average of 95 ±1% (85 to 100%), suggesting that in these neurons, the EPSCs were exclusively mediated by non-NMDA type of receptors.

The amplitude of the responses lacking a late component was smaller than the

early component of Types II and III responses but slightly larger than that of Type I (Table I). The range of response amplitudes of neurons lacking a late component largely overlapped with that of the early response of neurons expressing a late response. This indicates that the absence of a late response was not likely the result of deficient afferent fibers activation.

Neurons lacking a late response were most commonly encountered before P5 (16/45; 36%) or after P16 (24/33; 73%), whereas they were only seldom encountered between P5 and P15 (14/165; 8%; Fig. 4D). After P 20, the EPSC comprised only an early component in 17 out of 19 responses recorded, showing that under the present conditions, very few neurons expressed NMDA receptor-mediated EPSC after the third postnatal week.

When the ratio between the late and early components of the EPSC was plotted as a function of postnatal age a clear developmental pattern emerged: the late to early response ratio increased up to around P12 and decreased afterwards to become virtually equal to zero during the $4^{th}$ postnatal week and adulthood (Fig. 5). These results indicate that the ratio between NMDA and AMPA/KA receptor-mediated current was largest during the second postnatal week whereas AMPA/KA receptor-mediated currents largely dominated the responses later during development and adulthood.

Figure 5.

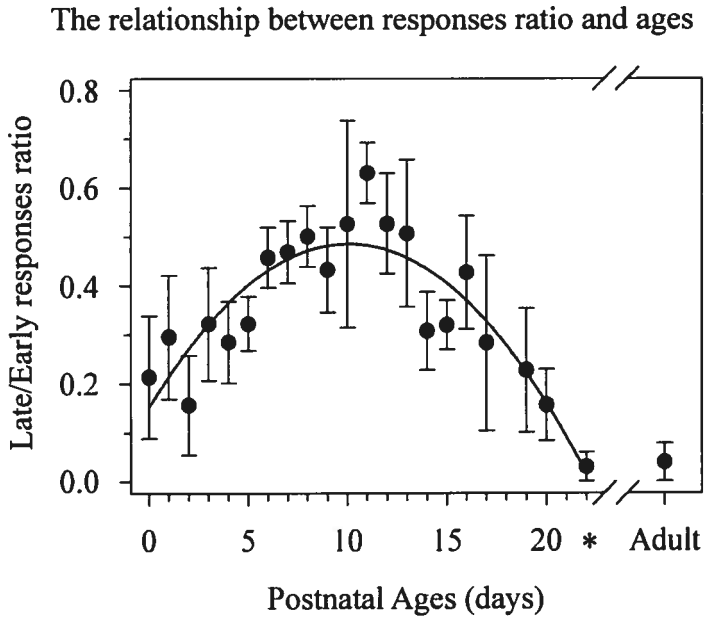


**Figure 5.** Ratio between the late and early components of the EPSCs as a function of postnatal age. A statistically significant 2nd degree polynomial correlation was found between response amplitude and postnatal age ($r^2$ = 0.784, $F_s$ = 32.660, $p<0.001$).

In addition to the ratio between the late and early components of the EPSC, other characteristics of EPSCs changed significantly over the first three to four postnatal weeks. The latency to peak of the early component of the EPSC substantially increased during the first four postnatal weeks and was even longer in adults (Fig. 6A). It averaged 8.8 ± 0.2ms (n = 105) during the first postnatal week and reached 13.8 ± 0.8ms (n=12) in adults, the increase being statistically significant ($T_s$ = 1239, $p<0.001$). These data have to be carefully interpreted for two reasons: the regression coefficient between postnatal age and response delay was small even though it was significant (Fig. 4A legend), and developmental factors explain only 4.6% of the variation in the delay. The increase in the decay time of the early response was slightly more robust, the regression explaining about 10% of the variation (Fig. 6B). This measure averaged 33 ± 1ms during the first postnatal week and increased steadily to reach 55 ± 6ms in the adult, a 22ms increase that was statistically significant ($t_s$ = 4.578, df = 115, $p<0.001$). In addition, the decay time of the early response was positively correlated with the amplitude of the response recorded at -100mV ($r = 0.218$, $F_s = 11.442$, $p < 0.001$), suggesting that part of the increase in duration of the response was due to an increase in its amplitude (see below), i.e., larger responses tended to have longer decay time. As a consequence of the increase in both early response peak latency and early response decay, the latency at which the late component of the EPSC was measured was significantly delayed with age (Fig. 6C) but yet sufficient enough to have a substantial incidence on the measurements of the late component because of its comparatively slow decay.

Figure 6.

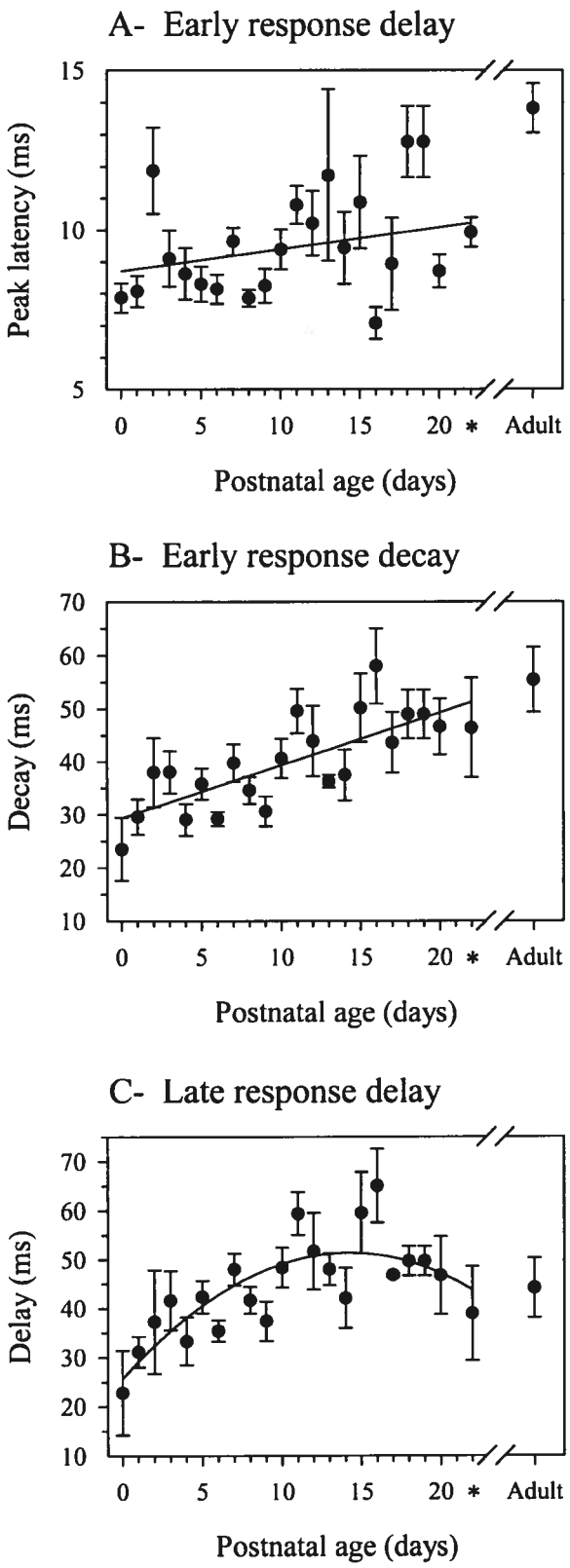


**Figure 6.** Characteristics of the early and late responses as a function of postnatal age. **A.** Time to peak of the early response from the electrical stimulus onset. A statistically significant linear correlation was found between time to peak and postnatal age ($r = 0.208$, $F_s = 10.315$, $p=0.0015$). **B.** Decay time from peak to a steady state plateau of the early response at an online membrane potential of -100mV. A statistically significant linear correlation was found between decay time and postnatal age ($r = 0.308$, $F_s = 23.962$, $p<0.001$). **C.** Delay between electrical stimulus onset and the steady state of the early response at which time the late response was measured. A statistically significant 2nd degree polynomial correlation was found between time delay and postnatal age ($r = 0.24$, $F_s = 13.998$, $p<0.001$). Each filled circle and bar represents the average ± SEM for neurons recorded on the corresponding postnatal day on the abscissa. Number of cells per postnatal day ranged from 2 to 26 with an average of 11.0 ± 1.5. No neurons were sampled on P18 and neurons from P21-27 ($n = 6$; labeled as * under the abscissa) and P59-71 ($n = 12$; labeled as Adult under the abscissa) were respectively pooled together. Total number of neurons was 243 in A, B and C.

In most cells, we could not detect the presence of a NMDA receptor-mediated component in the compound EPSCs in preparation from animals more than three weeks old. This is in contrast to several studies (e.g. Martin et al., 1999; Martin et al., 1997b; Martin et al., 1997a; Pennartz et al., 1991a) that reported the presence of NMDA receptor-mediated EPSCs in mature nAcb. To verify for the presence of NMDA receptor-mediated EPSCs in older animal, we have recorded EPSCs in preparations from animals during their fourth postnatal week with CNQX in the bathing medium. In all neurons tested using adequate stimulus parameter, we could record NMDA receptor-mediated EPSCs of amplitude comparable with those recorded in younger (Fig. 7A). Whereas EPSCs were comparable in amplitude, those recorded during the fourth postnatal week were found to be much shorter than those recorded during the second postnatal week (Fig. 7A right panel and 7B). Despite the fact that a substantial NMDA receptor-mediated EPSC could be evoked in the presence of CNQX, the presence of an NMDA component could not be clearly identified in the compound EPSC (Fig. 7C).

***Morphology***

A total of 310 neurobiotin filled MS neurons were recovered from the present and other series of similar experiments (Zhang and Warren, 1999, 2002). In neonatal rats, MS neurons appeared quite different from those described in adult animals. During the first 10 postnatal days, the dendrites appeared relatively thin, varicose and almost completely aspiny (Figs. 8A and 8B). The slenderness of the dendrites was particularly marked during the first few postnatal days during which the dendrites were so thin that they could be barely detected between the small varicosities (Fig. 8A). By the end of the first postnatal week, dendrites still had a varicose appearance but both the varicosities and

Figure 7.

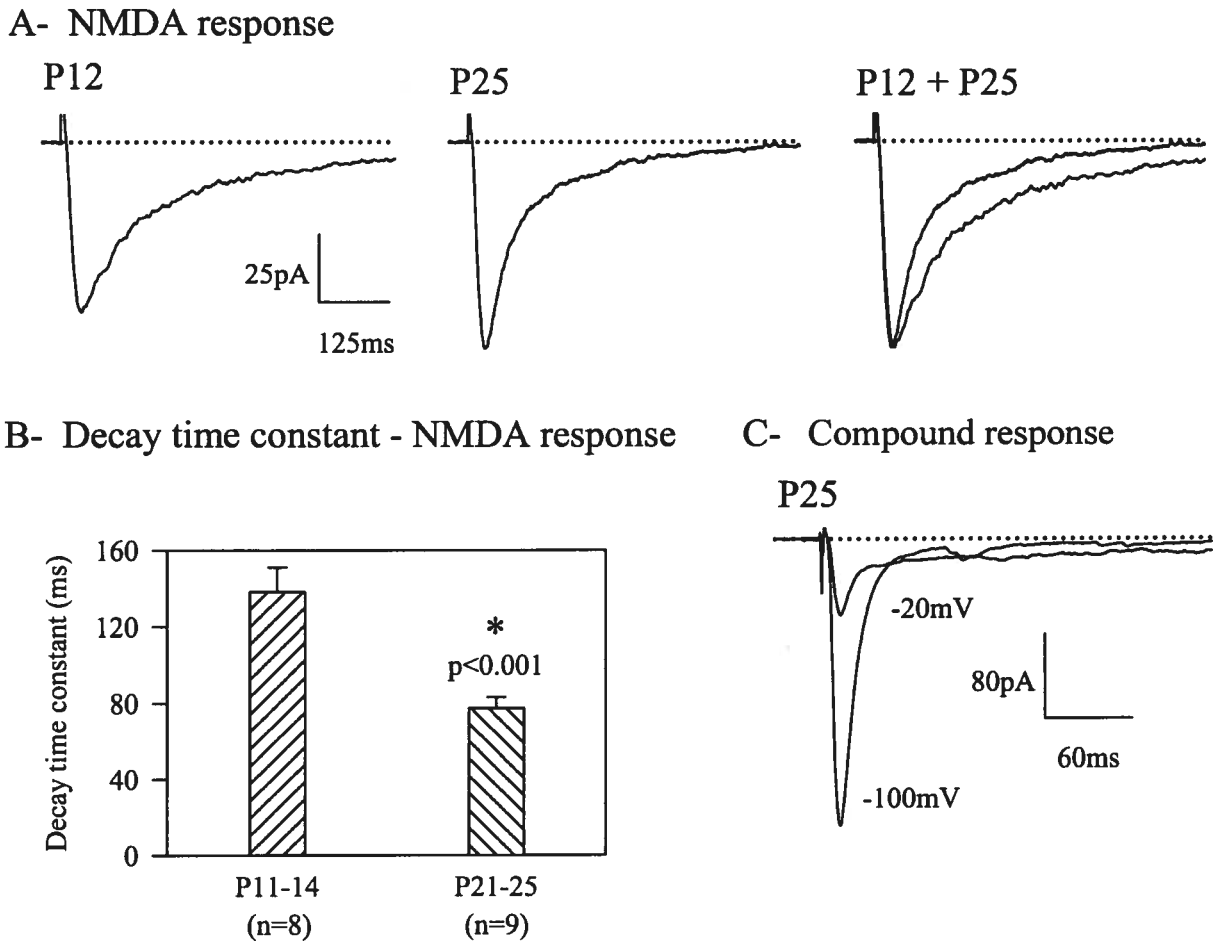


**Figure 7. A.** NMDA receptor-mediated EPSCs recorded at a holding membrane potential of -20mV in preparations from P12 (left panel) and P25 (middle panel) animals with CNQX (20μM) and BMI (10μM) present in the bathing medium. The right panel shows the overlay of the responses scaled on the amplitude of the P25 response. **B.** Average decay time constant of isolated NMDA receptor-mediated EPSCs recorded during the second (n=8) and fourth postnatal week (n=9). The time constant was measured by fitting a single exponential on the decay phase of the EPSCs. **C.** Compound response from the same P25 neuron shown in A at holding membrane potentials of -100 and -20mV in the absence of CNQX. Note the absence of an obvious NMDA receptor-mediated component at -20mV.

dendritic shafts appeared larger (Fig. 8B). During the second postnatal week, the dendrites gradually lost their varicose appearance and by P14, the dendrites of the majority of neurons unbroken continuous appearance (Fig. 8C) and cell displaying an immature appearance were rarely encountered during the third postnatal week and never afterwards (Fig. 8D). In addition, dendrites of more mature cells (e.g. Fig. 8C and 8D) were more convoluted than at earlier stages (e.g. Fig. 8A and 8B) and the dendritic field appeared to growth slightly during the first three postnatal weeks although we did not measure it.

We did not observed dendritic spines on highly varicosed dendrites but they were consistently present on dendrites from P12 and older neurons. Their density was very low at the beginning but appeared to increase until the end of period studied (P31) without apparently reaching a density comparable to the one found in adult MS neurons. The axon was also labeled in several neurons and it could often be followed for several hundreds μm (Fig. 8C and 8D, arrows). In these cases, it always branched within the nAcb before a single branch was seen leaving the nucleus and this even at the younger ages.

The cell body of MS neurons increased in size during the postnatal period covered by the present study as shown by the positive correlation between cell body perimeter and area and postnatal age (Fig. 9A and 9B). The somatic area increased by 24% between the first and third postnatal week from an average of $126 \pm 5\mu m^2$ to $156 \pm 13\mu m^2$ ($t_s = 2.656$, $df = 174$, $p = 0.009$). In contrast, there was no change in the number of primary dendrites during the same period (Fig. 9C).

Figure 8.

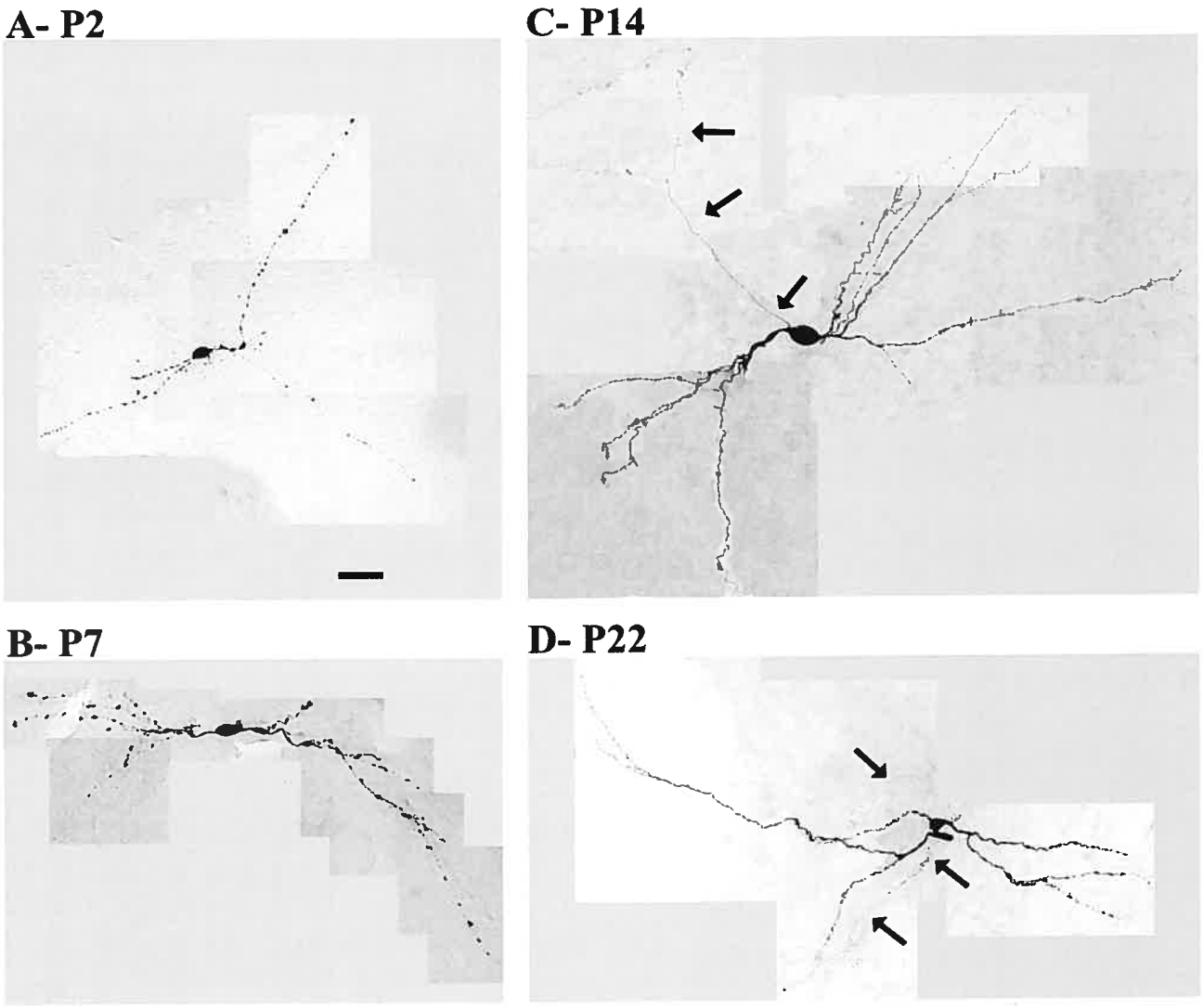


**Figure 8.** Image montages of neurobiotin filled neurons from P2 (A), P7 (B), P14 (C) and P22 (D) preparations. Arrows in panels C and D indicate the axons. The scale bar in panel A represents 50μm and applies to all panels.

Figure 9.

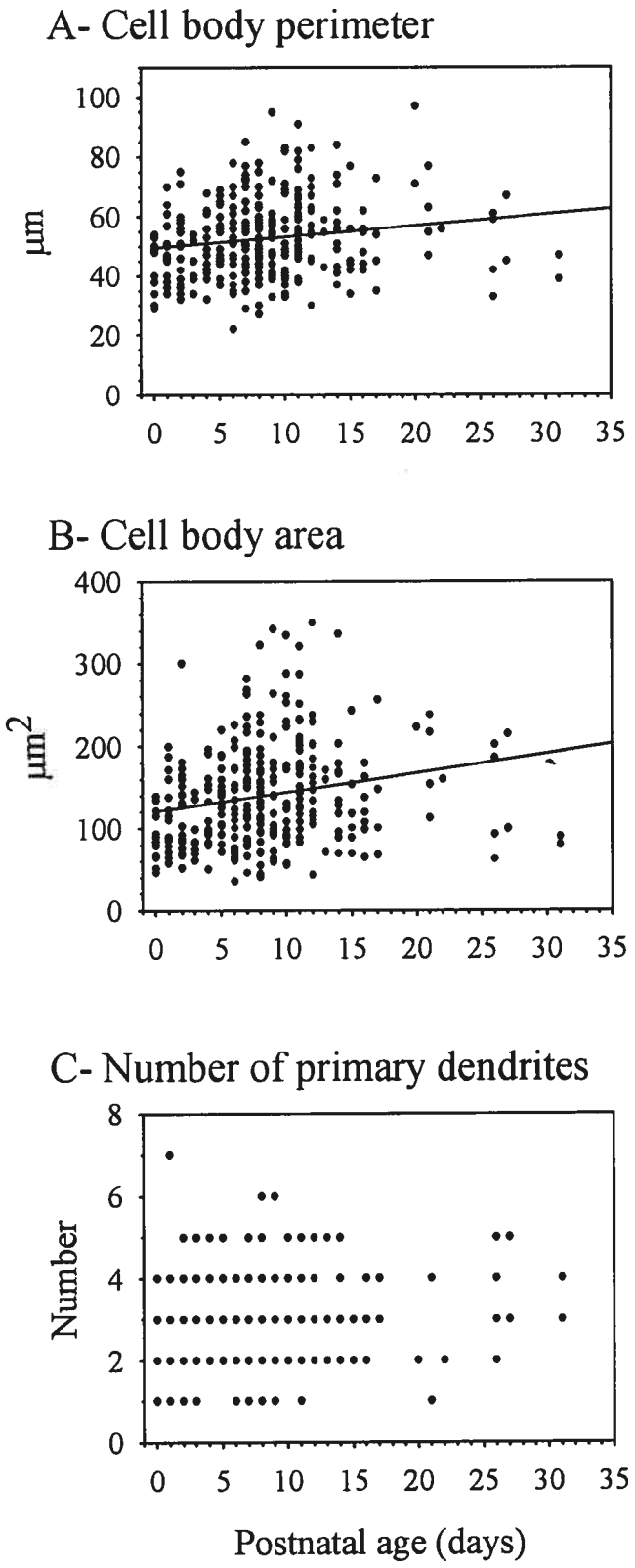


**Figure 9.** Graphs showing the cell body perimeter (**A**) and area (**B**) and the number of primary dendrites (**C**) of neurobiotin filled neurons as a function of postnatal age. Each dot represents one filled neuron and the straight line in A and B represents the linear regression. There was a significant correlation between cell body perimeter ($r = 0.192$, $p < 0.001$) and area ($r = 0.247$, $p < 0.001$) and postnatal age but not between the number of primary dendrites and postnatal age ($r = 0.069$, $p = 0.231$).

## DISCUSSION

We recorded electrically evoked EPSCs in nAcb neurons using slice preparations from rat pups (between P0 to P27) and young adult animals (P59 to P71). An EPSC was found in all recorded neurons (n = 243) demonstrating that functional excitatory synapses are already present in the nAcb at the time of birth. At all ages, the EPSCs were sensitive to glutamatergic antagonists, confirming that they were mediated by excitatory amino acids. Additionally, in a majority of neurons (189/243), the EPSC consisted of two distinct components: an early one with a peak between 3.6 and 21 ms after stimulus onset, and a late one that became apparent only at depolarized membrane potentials. The early and late components of the EPSC had $I_R$-$V_m$ relationships characteristic of AMPA/KA and NMDA receptor-mediated currents respectively, and were generally sensitive to specific receptor antagonists. However, in some cells from young preparations, both components could be abolished by only one type of antagonist.

During the first few days after birth, the amplitude of both AMPA/KA and NMDA receptor-mediated EPSCs was relatively small and then started to increase until the end of the second postnatal week. Whereas the amplitude of the AMPA/KA receptor-mediated response appeared to stabilize from that point on, the NMDA receptor-mediated response decreased rapidly during the following days and became virtually absent after the end of the third postnatal week. In addition, the ratio between the NMDA and AMPA/KA receptor-mediated responses followed a developmental pattern parallel to that of the NMDA receptor-mediated response, showing an increase during the first two postnatal weeks followed by a decrease. This was also reflected in the distribution of response types with age. Responses with comparatively large NMDA receptor-mediated

components were found mainly in preparations from P7 to P14 animals, whereas those showing small or no NMDA receptor-mediated components were more commonly found in preparations younger than P7 and older than P14. Together, these results show that there is a preeminent expression of NMDA receptor-mediated EPSC during the second postnatal week in the nAcb. NMDA receptor-mediated currents have been shown to play an important role during the activity-dependent developmental phase in several regions of the neuraxis (Murphy, 2003; Yoshimura et al., 2003; Savicc et al., 2003; Shibata et al., 2003; Schramm et al., 2002; Stegenga and Kalb, 2001; Luthi et al., 2001). The relative importance of NMDA receptor-mediated currents during the second postnatal week suggests that it could play a similar role in the nAcb during that time. The end of the second postnatal week corresponded also with the morphological maturation of MS neurons and the appearance of spines on their dendrites suggestive of intense synapse formation. This period also overlaps with the period of active maturation of membrane and firing properties of nAcb projection MS neurons (Belleau and Warren, 2000) implying a synchronization in the maturation of the input/output organization of the nAcb.

***Validity of the observations.*** Glutamatergic EPSCs could be evoked from the day of birth, showing that functional glutamatergic innervation of the nAcb is already underway before parturition. During the first two postnatal weeks, the amplitude of the EPSC increased suggesting an active maturation of nAcb glutamatergic innervation during that period. The fact that NMDA receptor-mediated currents decreased after the second postnatal week to be rarely observed after the third postnatal week, suggest that most of postsynaptic ionotropic glutamatergic neurotransmission in mature nAcb is mediated by

AMPA/KA type of receptors. The observation that the peak amplitude and duration of AMPA/KA-mediated currents during the 3rd and 4th postnatal weeks did not statistically differ from that recorded in adults does not necessarily imply that glutamatergic innervation of the nAcb is mature at this time. Further maturation and consolidation of the innervation may occur without significant changes in the amplitude of the response by a process involving an equilibrium between synapse formation and pruning.

In some neurons, both the early and late components of the response were abolished by APV, suggesting that the EPSC was mediated only by NMDA receptors. In addition, these results suggest that part of the NMDA receptor-mediated response recorded at hyperpolarized membrane potentials was less sensitive to $Mg^{2+}$ block. This is in agreement with the finding that the subunit composition of NMDA receptors is developmentally regulated and that NR2D subunits relatively resistant to $Mg^{2+}$ block are predominantly expressed during early postnatal stages (Ben Ari et al., 1988; Dunah et al., 1996; Kirson et al, 1999; Kleckner and Dingledine, 1991; Kuner and Shoepfer, 1996; Laurie et al., 1997; Monyer et al., 1994; Morrisett et al., 1990; Pollard et al., 1993; Wenzel et al., 1996). In some neurons, CNQX abolished both the early and late component of the response. These effects may have been mediated by an action of CNQX on the glycine binding site of the NMDA receptor (Lester et al., 1989; Pellegrini-Giampietro et al., 1989).

We did not pharmacologically isolate each of the two components of the EPSC in all neurons to measure isolated AMPA/KA and NMDA receptor-mediated currents. Our results suggest that there was a significant contribution by NMDA receptor-mediated currents to the early component of the response at depolarized membrane potentials. This

problem was circumvented by measuring the AMPA/KA receptor-mediated current at a hyperpolarized potential when the NMDA receptor-mediated current was minimal. Similarly, we measured the late component only after the early response had decayed in order that our data exclusively represented NMDA receptor-mediated responses, although with this method it was not possible to measure the peak of the NMDA receptor-mediated current. But the decay time of the late component of the response was very long and the NMDA response was very close to its peak at the time measurements were made (e.g. Fig. 2). We can conclude that our measurements represent fair representation of the amplitude of AMPA/KA and NMDA receptor-mediated postsynaptic currents in the nAcb.

Glutamatergic neurotransmission in the nAcb originates exclusively from external structures including the medial prefrontal cortex, the subiculum and the amygdala (Kelley et al., 1982; Groenewegen et al., 1987; Phillipson and Griffiths, 1985; Groenewegen et al., 1982; Krayniak et al., 1981; Groenewegen et al., 1980; Newman and Winans, 1980; Kelley and Domesick, 1982; Jayaraman, 1985). We have used local electrical stimulation to evoke glutamatergic EPSCs in the nAcb and presumably, we have indiscriminately activated afferents of various origins. Consequently, it is impossible to know if the innervation from different sources follows different developmental patterns and/or time courses. Alternatively, it is possible (although unlikely) that our results represent the postnatal development of one specific group of afferents. Nevertheless, the present results clearly show that at least one source of glutamatergic afferents involve transient expression of NMDA receptor-mediated EPSCs in developing nAcb. Future experiments should examine the development of individual afferents.

It is likely that the pattern of change in NMDA receptor-mediated currents that we found reflect an actual weakening in NMDA receptor-mediated current occurring in maturing nAcb. This is supported by the fact that while the NMDA receptor-mediated component of the EPSC decreased, the AMPA/KA receptor-mediated response remained constant, ruling out the possibility that the change was due to improper stimulation of afferents or a decrease in the density of the innervation. In addition, the fact that there was a strong correlation between the amplitude of the early and late components, combined with the stability of the early response, also suggest a weakening of the NMDA receptor-mediated current with aging. This is in agreement with other studies reporting that NMDA receptor-mediated responses were weak in preparations of the nAcb from young adults *in vitro* under normal conditions (Pennartz et al., 1991b; Martin et al., 1997b; Martin et al., 1997a). Substantial NMDA receptor-mediated responses could only be recorded by using high stimulus intensity and paired-pulse stimulation in conditions where inhibition was reduced (Martin et al., 1997a; Rajadhyaksha et al., 1998) underlying the weakness of NMDA receptor-mediated postsynaptic currents in adult nAcb. The difference between developing and mature animals suggest that NMDA receptors play different roles in young and adult animals, participating actively in fast glutamatergic neurotransmission at early developmental stages and being confined to a neuromodulatory role at later stages. Furthermore, there is much evidence that a substantial proportion of NMDA receptors are located on glutamatergic terminals rather than on nAcb postsynaptic neurons in mature nAcb (Grace, 1991).

The most striking change in MS neurons morphology was observed in individual dendrites which were essentially aspiny and highly varicose during the first 10-12

postnatal days. In addition, the soma of MS neurons slightly increased in size over the period studied. These finding are in close agreement with those observed in the dorsal striatum in the same specie (Tepper and Trent, 1993; Tepper et al., 1998). In the dorsal striatum, the third postnatal week corresponds with the period during which there is the greatest increase in axospinous asymmetric synapses (Hattori and McGeer, 1973; Tepper et al., 1998), a period corresponding with the relative decline in NMDA receptor-mediated EPSC in the present study.

***Functional significance***

The gross development of the brain is genetically guided. In contrast, the fine tuning of synaptic organization is often activity-dependent, allowing the individual to adapt to the prevailing environment. It is now well accepted that some fine tuning of neuronal connectivity also occurs in mature organisms but for the most part, it occurs during postnatal developmental stages and often irreversible.

The glutamatergic innervation of the nAcb has its origin in several preponderant limbic structures although the exact timing of arrival of the fibers from those locations is not known. We can assume that there will be some competition/cooperation among these different inputs that will presumably follow Hebbian rules (Hebb, 1949). The involvement of NMDA receptor-mediated currents in such mechanisms has been recognized for a number of years (Collingridge and Singer, 1990) and they could play a similar role during nAcb development.

The different sources of glutamatergic innervation of the nAcb are known to each have specific functions and to be active under specific behavioral circumstances related to environment. Presumably, depending upon the environmental context during synapse

formation, some of these inputs might be over active during a critical period and produce an imbalance between the different inputs, eventually resulting in a pathological state at later stages. Recent studies in which an imbalance between the different glutamatergic inputs to the nAcb have been produced by lesioning specific pathways at P7 have resulted in behavioral changes in the adult that were accompanied by biochemical changes in the nAcb during adulthood (Flores et al., 1996a; Flores et al., 1996b; Lipska et al., 1993; Weinberger and Lipska, 1995; Lipska et al., 1998). This suggests that such processes might be involved in pathological states.

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# CHAPTER III

**RÉSUMÉ**

Nous avons étudié les effets modulateurs des agonistes cholinergiques sur les courants postsynaptiques excitateurs (CPSEs) dans des neurones du noyau accumbens (nAcb) pendant le développement postnatal. Les enregistrements ont été obtenus dans des tranches provenant des rats âgés de un jour (jour postnatal 1, P1) à P27 en utilisant la technique *whole-cell patch-clamp*. Les CPSEs ont été produits par une stimulation électrique locale, et toutes les expériences ont été réalisées en présence du *bicuculline methchloride* dans le milieu extracellulaire et avec du QX-314 dans la pipette d'enregistrement. Dans ces conditions, les courants postsynaptiques étaient composés de CPSEs glutamatergiques comprenant deux composantes médiés par des récepteurs AMPA/KA et de NMDA. L'addition d'acétylcholine (ACh) ou de carbachol (CCh) produisait une diminution de 30-60% des deux composantes des CPSEs. Par contre, l'ACh produisait une augmentation (≈35%) des CPSEs lorsqu'administrée en présence d'atropine; un antagoniste des récepteurs muscariniques. Les effets excitateurs de l'ACh en présence d'atropine ont pu être reproduits par un agoniste des récepteurs nicotiniques, l'iodure 1,1-diméthyl-4-phényl-piperazinium (DMPP), et pouvaient être bloqués par la mécamylamine, un antagoniste des récepteurs nicotiniques. Les effets antagonistes de l'atropine pouvaient être reproduits par le pirenzepine, suggérant que la dépression muscarinique des CPSEs implique des récepteurs muscariniques de type M1/M4. En outre, les effets inhibiteurs de l'ACh sur les CPSEs médiés par le récepteur NMDA ont augmenté significativement pendant les deux premières semaines postnatales alors que les effets sur les CPSEs AMPA/KA sont demeurés stables. Nous avons constaté que, dans les présentes conditions expérimentales, les agonistes cholinergiques ne produisaient

aucun changement dans la conductance membranaire, la constante de temps des CPSEs AMPA/KA ni sur les réponses évoquées par application de glutamate exogène en présence de tétrodotoxine. Par contre ils ont produit des changements sur le rapport des réponses à des stimuli pairés, suggérant que leur action était médiée principalement par des mécanismes présynaptiques. Lorsque le QX-314 a été omis de la solution interne de l'électrode d'enregistrement, le CCh a produit des changements dans le potentiel de repos des neurones et à augmenté le nombre de potentiels d'action produit par une injection intracellulaire de courant, suggérant que le QX-314 bloquait les effets cholinergiques postsynaptiques. Ensemble, ces résultats suggèrent que l'ACh peut diminuer ou augmenter la neurotransmission glutamatergique dans le nAcb en agissant respectivement sur les récepteurs muscariniques et nicotiniques situés sur les terminaisons glutamatergiques. La modulation cholinergique de la neurotransmission médiée par les récepteurs AMPA/KA et NMDA dans le nAcb pendant le développement postnatal pourrait jouer un rôle important dans les processus développementaux dépendant de l'activité.

**Mots-Clés** : Acétylcholine; récepteur muscarinique, récepteur nicotinique, courant excitateur postsynaptique, mécanisme présynaptique

# Muscarinic and Nicotinic Presynaptic Modulation of EPSCs in the Nucleus Accumbens During Postnatal Development

LIMING ZHANG[1,2] AND RICHARD A. WARREN[1,3]
[1]*Centre de Recherche Fernand-Seguin,* [2]*Department of Physiology and* [3]*Department of Psychiatry, University of Montréal, Montreal, Quebec H1N 3V2, Canada*

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**Zhang, Liming, and Richard A. Warren.** Muscarinic and nicotinic presynaptic modulation of EPSCs in the nucleus accumbens during postnatal development. *J Neurophysiol* 88: 3315–3330, 2002; 10.1152/jn.01025.2001. We have studied the modulatory effects of cholinergic agonists on excitatory postsynaptic currents (EPSCs) in nucleus accumbens (nAcb) neurons during postnatal development. Recordings were obtained in slices from postnatal day 1 (P1) to P27 rats using the whole cell patch-clamp technique. EPSCs were evoked by local electrical stimulation, and all experiments were conducted in the presence of bicuculline methchloride in the bathing medium and with QX-314 in the recording pipette. Under these conditions, postsynaptic currents consisted of glutamatergic EPSCs typically consisting of two components mediated by AMPA/kainate (KA) and *N*-methyl-D-aspartate (NMDA) receptors. The addition of acetylcholine (ACh) or carbachol (CCh) to the superfusing medium resulted in a decrease of 30–60% of both AMPA/KA- and NMDA-mediated EPSCs. In contrast, ACh produced an increase (≈35%) in both AMPA/KA and NMDA receptor-mediated EPSCs when administered in the presence of the muscarinic antagonist atropine. These excitatory effects were mimicked by the nicotinic receptor agonist 1,1-dimethyl-4-phenyl-piperazinium iodide (DMPP) and blocked by the nicotinic receptor antagonist mecamylamine, showing the presence of a cholinergic modulation mediated by nicotinic receptors in the nAcb. The antagonistic effects of atropine were mimicked by pirenzepine, suggesting that the muscarinic depression of the EPSCs was mediated by $M_1/M_4$ receptors. In addition, the inhibitory effects of ACh on NMDA but not on AMPA/KA receptor-mediated EPSC significantly increased during the first two postnatal weeks. We found that, under our experimental conditions, cholinergic agonists produced no changes on membrane holding currents, on the decay time of the AMPA/KA EPSC, or on responses evoked by exogenous application of glutamate in the presence of tetrodotoxin, but they produced significant changes in paired pulse ratio, suggesting that their action was mediated by presynaptic mechanisms. In contrast, CCh produced consistent changes in the membrane and firing properties of medium spiny (MS) neurons when QX-314 was omitted from the recording pipette solution, suggesting that this substance actually blocked postsynaptic cholinergic modulation. Together, these results suggest that ACh can decrease or increase glutamatergic neurotransmission in the nAcb by, respectively, acting on muscarinic and nicotinic receptors located on excitatory terminals. The cholinergic modulation of AMPA/KA and NMDA receptor-mediated neurotransmission in the nAcb during postnatal development could play an important role in activity-dependent developmental processes in refining the excitatory drive on MS neurons by gating specific inputs.

## INTRODUCTION

The nucleus accumbens (nAcb) constitutes the major portion of the ventral striatum and is an important point of convergence of information originating in several limbic structures, including the prefrontal cortex (PFC), the amygdala, the hippocampus, and the midline thalamic nuclei (Groenewegen et al. 1980, 1982, 1987; Jayaraman 1985; Kelley and Domesick 1982; Kelley and Stinus 1984; Kelley et al. 1982; Krayniak et al. 1981; Newman and Winans 1980; Phillipson and Griffiths 1985). These projections, believed to be mainly glutamatergic, are thought to mediate their excitatory drive by acting on *N*-methyl-D-aspartate (NMDA) and AMPA/kainate (KA) glutamatergic receptors (DeFrance et al. 1985; Finch 1996; Kombian and Malenka 1994; Nicola et al. 1996; ;Yim and Mogenson 1982 Zhang and Warren 1999). The primary output of the nAcb is to the ventral pallidum (Hakan et al. 1992; Yang and Mogenson 1985), which is involved in the activation of voluntary movements (Heimer et al. 1994; Swerdlow and Koob 1987). This input/output organization suggests that the nAcb is an important interface between motivational and motor systems driven by the ventral pallidum (Beninger et al. 1983; Lopes da Silva et al. 1984; Mogenson et al. 1980). The nAcb is known to be involved in reinforcement aspects of behavior (Cador et al. 1991; Joseph and Hodges 1990; Wise and Bozarth 1987) and could be implicated in a number of psychiatric diseases, such as schizophrenia (Csernansky et al. 1991; Grace 1992; Matthysse 1983; Snyder 1973) and Tourette's syndrome (Braun et al. 1993; Comings 1987).

The only class of neurons that project outside the nAcb are the medium spiny (MS) neurons, which are GABAergic and account for about 95% of the neuronal population. In addition, the nAcb contains small populations of interneurons including the large aspiny (LA) neuron, which is the only known source of acetylcholine (ACh) in the nAcb (Meredith and Chang 1994; Meredith and Wouterlood 1990; Meredith et al. 1989; Phelps et al. 1985). Cholinergic systems have been implicated in fundamental aspects of human behavior including memory, motivation, and motor behavior (File et al. 1998; Gotti et al. 1997). Interest in understanding cholinergic mechanisms involved in the control and regulation of motor and higher brain functions has been growing ever since the neostriatal cholinergic system

Address for reprint requests: R. A. Warren, Centre de Recherche Fernand-Seguin, 7331 Hochelaga St., Montréal, Québec H1N 3V2, Canada [redacted].

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was postulated to play a role in the pathophysiology of several diseases. Alterations in the levels of ACh and cholinergic receptors have been linked to neurological and neuropsychological diseases including schizophrenia and Parkinson's disease (Gotti et al. 1997; Lena and Changeux 1997; MacDermott et al. 1999). Selective loss of cholinergic neurons in the nAcb in schizophrenia and Alzheimer's disease has also been demonstrated (Holt et al. 1999; Lehéricy et al. 1989).

In the nAcb, LA neurons establish synaptic contacts with MS neurons (Contant et al. 1996) as well as with glutamatergic terminals (Meredith and Wouterlood 1990). The action of ACh is mediated by nicotinic and muscarinic receptors, which are both present in substantial amounts in the nAcb and dorsal striatum (Bernard et al. 1992; Clarke et al. 1984; Court and Perry 1995; Hersch and Levey 1995; Hersch et al. 1994; Levey et al. 1991; Schliebs and Robner 1995). Consistent with the cellular location of cholinergic muscarinic receptors (Wei et al. 1994), ACh has been found to modulate glutamatergic neurotransmission in MS neurons by acting on presynaptic muscarinic receptors (Pennartz and Lopes da Silva 1994; Sugita et al. 1991) and to increase the excitability of MS neurons by acting on muscarinic postsynaptic receptors (Sugita et al. 1991; Uchimura and North 1990). The role of nicotinic receptors in modulating the activity of MS neurons has not been investigated.

It has been proposed that the major role of nicotinic cholinergic receptors in the CNS, including the nAcb, is to modulate synaptic transmission by controlling neurotransmitter release rather than by exerting direct postsynaptic actions (Gray et al. 1996; MacDermott et al. 1999; McGehee et al. 1995; Wonnacott 1997). Nicotine has been found to facilitate the release of diverse neurotransmitters, including GABA (Guo et al. 1998; Léna et al. 1993), glutamate (Fisher and Dani 2000; Girod et al. 2000; Guo et al. 1998; McGehee et al. 1995; Radcliffe and Dani 1998; Toth et al. 1993), ACh (McGehee et al. 1995), dopamine (Auta et al. 2000; Puttfarcken et al. 2000; Rapier et al. 1988, 1990; Sharples et al. 2000), and 5-HT (Reuben and Clarke 2000). Whereas nicotine can enhance glutamatergic neurotransmission, it has also been found to differentially modulate AMPA/KA and NMDA receptor-mediated synaptic transmission (Aramakis and Metherate 1998). In the striatum, including the nAcb, nicotine has been found to increase neuronal glutamate release (Kaiser and Wonnacott 2000; Reid et al. 2000; Toth et al. 1992, 1993). The presence of nicotinic receptors on glutamatergic terminals in the nAcb is also supported by the fact that glutamatergic neuronal populations known to project to the nAcb express high levels of several nicotinic receptor subunit mRNAs, whereas a comparatively low expression of these subunits is found in the nAcb itself (Quik et al. 2000; Wada et al. 1989, 1990).

The goal of the present study was to understand how ACh, through an action on both muscarinic and nicotinic receptors, modulates glutamatergic neurotransmission in the nAcb. Our findings suggest that ACh acts on both muscarinic and nicotinic presynaptic receptors to modulate glutamatergic neurotransmission, but whereas muscarinic receptor activation depresses excitation, nicotinic receptor activation enhances glutamatergic neurotransmission. Parts of the present study have appeared in abstract form (Zhang and Warren 2000).

## METHODS

### *Slice preparation*

The procedures used to obtain nAcb slice preparation have been described elsewhere (Belleau and Warren 2000). Briefly, 400-$\mu$m parasagittal slices containing the nAcb were obtained from rat pups on the day following birth (P1) up to P27. Slices were incubated for at least 1 h before recording was undertaken in a submerged-type chamber superfused with room temperature (22–25°C) artificial cerebrospinal fluid (ACSF) containing (in mM) 126 NaCl, 26 $NaHCO_3$, 10 dextrose, 3 KCl, 1.3 $MgSO_4$, 2.5 $CaCl_2$, and 1.25 $NaH_2PO_4$ with a pH of 7.4 when bubbled with a gas mixture of 95% $O_2$-5% $CO_2$. The nAcb was visualized with a stereo microscope using the anterior commissure, the neostriatum, the septum, and the ventricles as landmarks based on Paxinos and Watson (1986).

### *Recording*

Whole cell recording was achieved using the blind patch-clamp technique (Blanton et al. 1989). Pipettes were pulled from thin wall borosilicate capillary glass with a P-87 micropipette puller (Sutter Instrument). The pipettes had a resistance of 3–5 M$\Omega$ when filled with a solution containing (in mM) 140 potassium gluconate, 2 $MgCl_2$, 0.1 $CaCl_2$, 1.1 EGTA, 10 HEPES, 2 $K_2$-ATP (ATP), and 0.5 guanosine trisphosphate (GTP). Biocytin (0.3%) and QX-314 (2 mM; Alomone Labs) were routinely added to the recording solution to label recorded neurons and to minimize voltage-sensitive $Na^+$ channels generating action potential, respectively. The pH of the recording solution was adjusted to 7.3 with 8N KOH solution, and its final osmolarity was adjusted to 285–290 mosmol/kg. Neurons were recorded in continuous single-electrode voltage-clamp mode with an Axoclamp 2B amplifier (Axon Instruments). The output of the amplifier was fed to a LPF 200A DC amplifier/filter (Warner Instruments) and digitized at 0.5–10 kHz with a real-time acquisition system Digidata 1200 (Axon Instruments). Data acquisition was achieved using the pClamp 6.0 software (Axon Instruments), and off-line analysis was performed with pClamp 6.0 and Cambridge Electronic Design softwares. The resting membrane potential (RMP) was measured as soon as the whole cell configuration was achieved, and the offset potential, measured on withdrawal of electrode from the cell, was accounted for assuming that it drifted in a linear fashion with time from the start of the recording session. A −10-mV correction for liquid junction potential was routinely added to membrane potential measurements (Spigelman et al. 1992).

### *Synaptic stimulation*

Excitatory postsynaptic currents (EPSCs) were evoked by means of a monopolar tungsten microelectrode placed close to the border of the nAcb, 0.5–1.0 mm away from the recording electrode. The stimuli consisted of single 0.1-ms, 3- to 6-V cathodal pulses delivered at 15-s intervals. Paired-pulse stimulation with the same parameters and separated by 50 ms were used in some experiments to distinguish between pre- and postsynaptic mechanisms. All experiments were performed with bicuculline methochloride (BMI) 10 $\mu$M present in the superfusing medium solution to block $GABA_A$ receptor-mediated synaptic currents and to isolate glutamatergic-mediated EPSCs (Zhang and Warren 1999). Under these conditions, the addition of glutamatergic antagonists completely abolished synaptic responses (e.g., Fig. 1), and in no cases did we observed evidence that the stimulus directly activated the neuron under study. In all experiments, the membrane potential was clamped on-line at −70 mV, and the EPSCs were recorded at potentials between −100 and +40 mV using incremental steps of 20 mV.

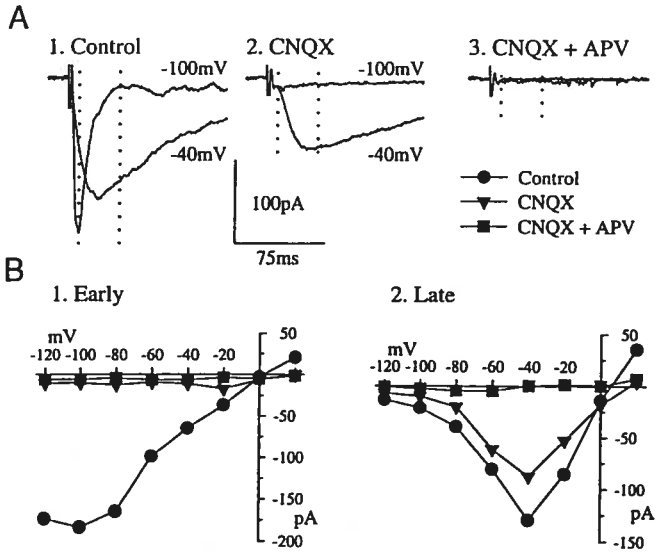


FIG. 1. Nature of the excitatory postsynaptic current (EPSC) evoked by local electrical stimulus in the presence of bicuculline methochloride (BMI, 10 $\mu$M). *A*: current traces of the response evoked by single local electrical stimulus and recorded at holding membrane potentials of −40 and −100 mV before glutamatergic antagonists application (*1*) and during superfusion with 6-cyano-7-nitroquinoxalene-2,3-dione (CNQX; 20 $\mu$M; *2*) and CNQX and 2-amino-5-phosphonovaleric acid (APV; 50 $\mu$M; *3*). Recordings were obtained in a medium spiny (MS) neuron from a P3 animal. Current traces represent the average of 8 sweeps. *B*: current-voltage relationship of the response ($I_R$-$V_m$) between −120 and 20 mV. The early component was measured 9 ms after the stimulus as indicated in *A*, left vertical dotted line. The late component was measured 43 ms after the stimulus as indicated in *A*, right vertical dotted line.

### *Pharmacological agents*

The following pharmacological agents were used: 6 cyano-7-nitroquinoxaline-2,3-dione (CNQX; 20 $\mu$M), (+)-2-amino-5-phosphonopentanoic acid (APV; 50 $\mu$M), carbachol (CCh; 50 $\mu$M); ACh (100 $\mu$M); atropine sulfate (10 $\mu$M); pirenzepine HCl (10 $\mu$M); 1,1-dimethyl-4-phenyl-piperazinium iodide (DMPP; 10 $\mu$M); mecamylamine HCl (MMA; 10 $\mu$M). CNQX and APV were obtained from Tocris Cookson; CCh, atropine, pirenzepine, DMPP, and mecamylamine were from Research Biochemicals International; and ACh was from Sigma. Drugs, with the exception of CNQX, which was dissolved in dimethlysulfoxide (DMSO), were made up as 10 mM stock solutions in distilled water (ACh on the day of use) and diluted with external solution to final concentration just before their addition to the perfusion medium. The final concentration of DMSO during CNQX administration was 0.1%. Under our experimental conditions, the full effect of cholinergic agonists on the response occurred 5–7 min following their addition to the bathing medium and no recording was made before a drug had been perfused for at least 15 min. Antagonists were added to the superfusing medium at least 15 min. and then a baseline was recorded before the addition of agonists. In several cases atropine was present in the ACSF throughout the experiment. The slice was superfused with control ACSF for at least 30 min to allow washout of a drug before a new baseline was recorded. In some experiments, slices were incubated for 2 h in the presence of the antagonist (mecamylamine) prior to experimentation to enable full penetration of the drug into the slice. Synaptic currents were stored as the on-line average of four to eight events at each membrane potential before, during, and after drug administration.

### *Statistics*

Statistical analysis was performed with SigmaStat software (SPSS) using paired Student's *t*-test to compare the response before and during the application of agonists and antagonists. Probability values of $<0.05$ were considered statistically significant. All numerical data are expressed as means ± SE. Neurons that could not be unambiguously classified as MS based on their physiological characteristics (Belleau and Warren 2000) and morphological appearance were excluded from statistical analysis.

## RESULTS

Whole cell voltage-clamp recording was obtained from 127 MS neurons in slices from rats between P1 and P27. Most cells ($n$ = 86) were recorded in preparations from P5 to P15 animals, a time frame during which relatively large NMDA-mediated responses can be more readily evoked (Zhang and Warren 1999). The membrane and firing characteristics of MS neurons were similar to those previously reported for animals of comparable age (Belleau and Warren 2000). In addition, 79 neurons filled with biocytin were examined under light microscopy and displayed features that have been previously attributed to MS neurons from animals of similar age (Tepper et al. 1998).

### *Characteristics of glutamatergic EPSCs*

Typically, postsynaptic currents evoked by local electrical stimulation in the presence of the $GABA_A$ receptor antagonist BMI consisted of a compound glutamatergic EPSC comprising an early and a late component mediated, respectively, by the activation of AMPA/KA and NMDA receptors (Fig. 1). We characterized postsynaptic EPSCs in 91 neurons; the EPSC in 79 displayed an early and a late component, whereas only an early component was found in the remaining 12.

The early EPSC peaked between 3.6 and 21 ms. after stimulus onset at a holding membrane potential of −100 mV, had a linear relationship with the membrane potential and reversed around 0 mV ($n$ = 91). In contrast, the maximal amplitude of the late EPSC occurred much later, was usually observed at holding membrane potentials of −20 or −40 mV, displayed a nonlinear relationship with voltage, and also reversed around 0 mV ($n$ = 79).

Figure 1 shows a representative example of an EPSC recorded in a preparation from a P3 animal on which specific glutamatergic antagonists were tested. During the control period (Fig. 1*A1*), the early EPSC peaked 9 ms. after the stimulus onset at a holding membrane potential of −100 mV, and the response decayed to baseline within 35 ms. The current voltage relationship ($I_R$-$V_m$) of the early EPSC was linear at membrane potentials between −80 and 20 mV, but the response appeared to saturate at membrane potentials below −80 mV (Fig. 1*B1*). Bath application of the AMPA/KA receptor antagonist CNQX completely abolished the early component of the EPSC, and there was virtually no residual postsynaptic current at all membrane potentials at the latency the early response was measured (Fig. 1, *A2* and *B1*).

The late component, measured after the early component had decayed, increased at membrane potentials between −100 and −40 mV and reached its maximum usually at −40 or −20 mV. At more depolarized membrane potentials, it decreased and reversed polarity around 0 mV (Fig. 1, *A* and *B, 2*), a current-voltage relationship typical of NMDA receptor-mediated current. The further addition of the NMDA receptor antagonist APV to the superfusing medium completely abol-

ished the late EPSC (Fig. 1*A3*), demonstrating that it was mediated by NMDA-type receptors. In the presence of CNQX alone, the NMDA receptor-mediated EPSC was recorded in isolation showing that measurements of the late component of the EPSC made on the compound EPSC were close to the peak of the NMDA-mediated EPSC and represented mostly NMDA receptor-mediated current (Fig. 1*A2*). Also, note there was no residual postsynaptic current in the presence of CNQX and APV, showing that glutamatergic EPSCs were effectively isolated by the addition of BMI to the superfusing medium. CNQX and APV were tested together in four other neurons producing similar results. In addition, CNQX and APV were tested individually in 17 and 14 neurons, respectively, producing an inhibition of the early and late components of the response of 91 ± 2 and 85 ± 5%.

In most neurons, the effects of cholinergic agonists and antagonists were assessed at holding membrane potentials usually between −100 and 40 mV in steps of 20 mV. The AMPA/KA-mediated EPSC was measured at the peak of the early component of the EPSC at a holding membrane potential of −100 mV, when the amplitude of the late component was minimal (Fig. 1*A*, left vertical dotted lines), whereas the effects on NMDA receptor-mediated currents were measured at a latency at which the early component recorded at a holding membrane potential of −100 mV had decayed (Fig. 1*A*, right vertical dotted lines).

### *Effects of cholinergic agonists*

The addition of the general cholinergic agonists ACh or CCh to the superfusing medium in the presence of BMI typically produced a decrease of both the early and late components of the EPSC. A representative example of this effect is shown in Fig. 2*A*. In this case, the amplitude of the early and late component of the EPSC recorded at −100 and −20 mV, respectively, was reversibly reduced by 38 and 40% during the application of CCh. Similar results were observed in 15 other neurons, while CCh produced no effects on the EPSC in one case. The effects of CCh on the early and late components of the EPSC as a function of holding membrane potential are summarized in Fig. 2*B*. The amplitude of the early component of the EPSC was significantly reduced at holding membrane potentials between −100 and −20 mV by an average of 39–46% ($n$ = 16). The magnitude of the effect of CCh on the early component of the EPSC did not vary significantly with holding membrane potential ($F_s$ = 0.220, $P$ = 0.926, df = 4,). No significant changes were observed at more positive membrane potentials because the EPSCs were small and the amplitudes were more variable. CCh also produced a reduction of

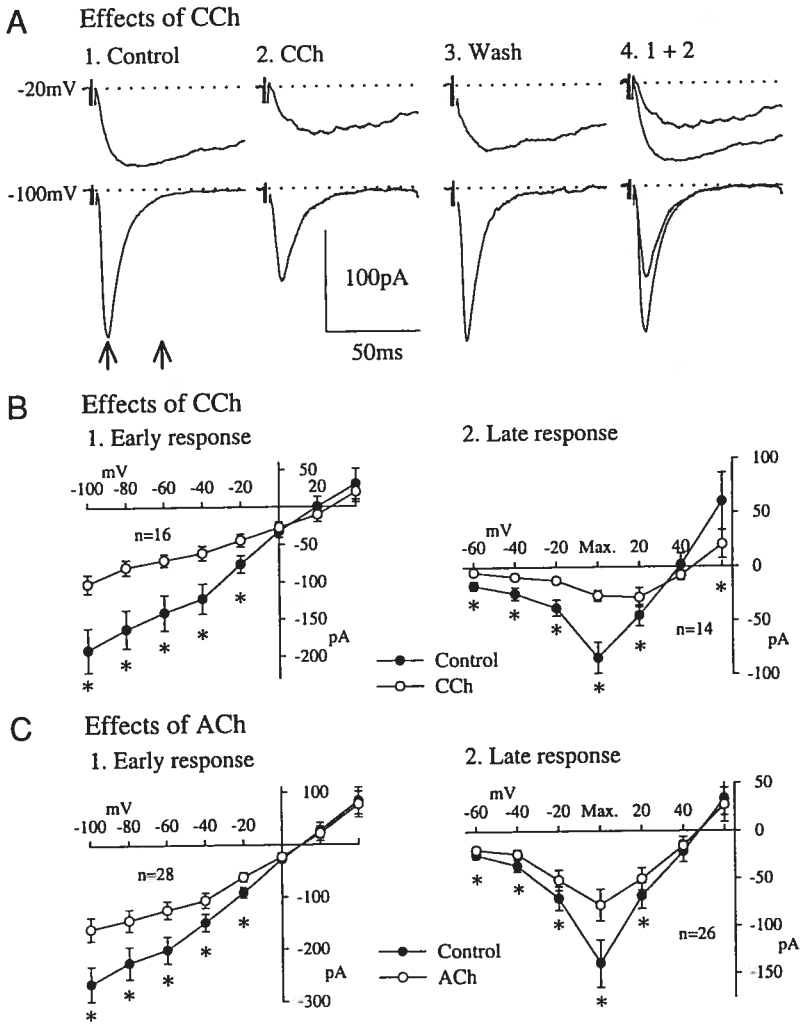


FIG. 2. Effect of cholinergic agonists on the EPSC. *A*: current traces of the response evoked by single local electrical stimulus recorded at holding membrane potentials of −20 and −100 mV before (*1*), during (*2*), and after (*3*) superfusion with carbachol (CCh, 50 μM). *4*: the overlay of the responses before and during CCh application. Current traces represent the average of 8 sweeps. BMI (10 μM) was present in the superfusing medium throughout recording. Recordings were obtained from a MS neuron in a preparation from a P8 animal. Left and right vertical arrows in *1* indicate where the early and late responses were respectively measured. *B*: average $I_R$-$V_m$ of the early ($n$ = 16; *1*) and late ($n$ = 14; *2*) recorded before and during superfusion with CCh. *C*: average $I_R$-$V_m$ of the early ($n$ = 28; *1*) and late ($n$ = 26; *2*) components of the EPSC recorded before and during superfusion with acetylcholine (ACh). In 2 neurons tested with CCh and 2 tested with ACh, the EPSC consisted only of an early component, and these were included in the average of the early component. The $I_R$-$V_m$ of the late response were aligned on the holding membrane potential at which the response was maximum before averaging (usually at −20 or −40 mV). Asterisk indicates a statistically significant difference between control and agonist treatment at this holding membrane potential (Student's $t$-test, $P < 0.05$).

the late component of the EPSC, which generally appeared to be of larger magnitude than that observed on the early component, averaging 45–72% ($n$ = 14) at holding membrane potentials at which a statistically significant effect was observed. Indeed, the effect of CCh was statistically smaller on early responses recorded at −100 mV than on late responses measured at their maximal amplitude (inhibition of 45 ± 5 and 66 ± 6%, respectively; $t_s$ = 3.408, $P$ = 0.002, df = 28).

ACh (100 μM) was tested in 33 neurons; it produced a reduction of the EPSC in 28 cells and no change in the remaining 5. The inhibitory effects of ACh appeared smaller than those produced by CCh on the early component of the EPSC (Fig. 2*C1*), ranging from 29 to 38% ($n$ = 28), although the difference was not statistically significant at any membrane potential (0.418 ≥ $t_s$ ≤.667, 0.1 ≥ $P$ ≤ 0.678, df = 42). As observed for CCh, the magnitude of the effects of ACh did not vary significantly with holding membrane potential ($F_s$ = 0.700, $P$ = 0.594, df = 4). The effects of ACh on the late component of the EPSC ranged from 26 to 44% at membrane potentials at which a significant inhibition was observed (Fig. 2*C2*). No significant difference was found between the effect of ACh on the late component at the membrane potential at which the responses were largest and on the early component recorded at −100 mV of the EPSC (inhibition of 44 ± 4 and 38 ± 5%, respectively; $t_s$ = 0.866, $P$ = 0.396, df = 52), although the effect of ACh on the late component of the response was significantly smaller than that produced by CCh ($t_s$ = 2.779, df = 38, $P$ = 0.008; 44 vs. 68%, respectively) at the membrane potential at which the late EPSC was largest.

To validate our experimental assumption that the effects of cholinergic agonists on the early and late components of the EPSC accurately represented the effects on AMPA/KA and NMDA receptor-mediated EPSCs, we studied the effects of ACh on pharmacologically isolated AMPA/KA and NMDA mediated EPSC using APV (50 μM) and CNQX (20 μM), respectively. In these experiments, ACh produced an inhibition on AMPA/KA and NMDA receptor-mediated EPSCs of a magnitude comparable to the effects observed on the early and late components of the compound EPSC. The AMPA/KA receptor-mediated EPSC peak was reduced by 40–45% at membrane potentials between −100 and −20 mV ($n$ = 7; Fig. 3*A*) and the NMDA receptor-mediated current by 61% at the membrane potential at which the response was the largest ($n$ = 4; Fig. 3*B*), thus confirming the observations made on compound EPSCs. In general, the effects of CCh and ACh were fully reversible after 10–30 min of washing with control ACSF.

Together, these results indicate that the activation of cholinergic receptors results in a net depression of both AMPA/KA and NMDA receptor-mediated EPSCs in nAcb MS neurons, whereas in some cases, the inhibition appeared larger on the NMDA than on the AMPA/KA mediated response.

### *Effects of muscarinic receptor antagonists*

To identify the type of receptors mediating the inhibitory action of cholinergic agonists, ACh was administered along with specific cholinergic receptor antagonists. We first tested the effects of the general muscarinic receptor antagonist atropine (10 μM). When administered alone, atropine produced an increase in both the early and late components of the EPSC in

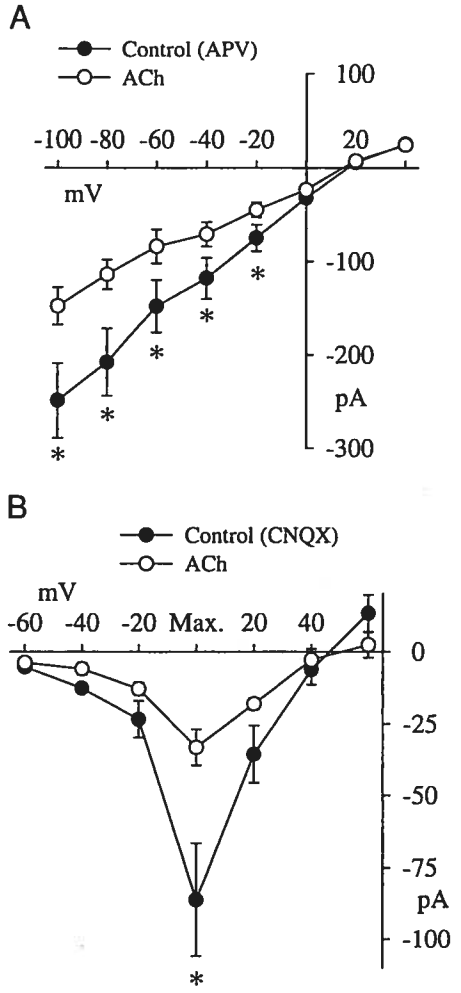


FIG. 3. *A*: average $I_R$-$V_m$ of the AMPA/KA receptor-mediated EPSC recorded in the presence of APV (50 μM) and BMI (10 μM) before (control) and during (ACh) the addition of ACh (100 μM) to the superfusing medium ($n$ = 7). *B*: average peak response of the *N*-methyl-D-aspartate (NMDA) receptor-mediated EPSC in the presence of CNQX (20 μM) and BMI (10 μM) before (control) and during (ACh) the addition of ACh (100 μM) to the superfusing medium ($n$ = 4). *, a statistically significant difference between control and agonist treatment at this holding membrane potential (Student's $t$-test, $P$ < 0.05).

five cells tested (Fig. 4*A*), suggesting that endogenous ACh produced a significant inhibition of the EPSC in our preparation. Interestingly, when concomitantly applied with atropine, ACh produced a further enhancement of the EPSC in most neurons tested instead of a decrease, as observed when general cholinergic agonists were administered alone.

Figure 4*B* shows an example of the effects produced by ACh administered in the presence and absence of atropine. In this case, atropine was first added to the superfusing medium for 15 min (Fig. 4*B1*) and, when ACh was added, a significant enhancement of both the early and late components of the EPSC was observed (Fig. 4*B2*). Following the washout of atropine, the same dose of ACh produced a significant decrease of the EPSC (Fig. 4*B3*) as compared with the atropine period and following the washout of ACh (Fig. 4*B4*).

The effects of ACh in the presence of atropine were tested in 18 neurons; a significant enhancement of the early component of the EPSC averaging 33% was observed in 13 (72%) neu-

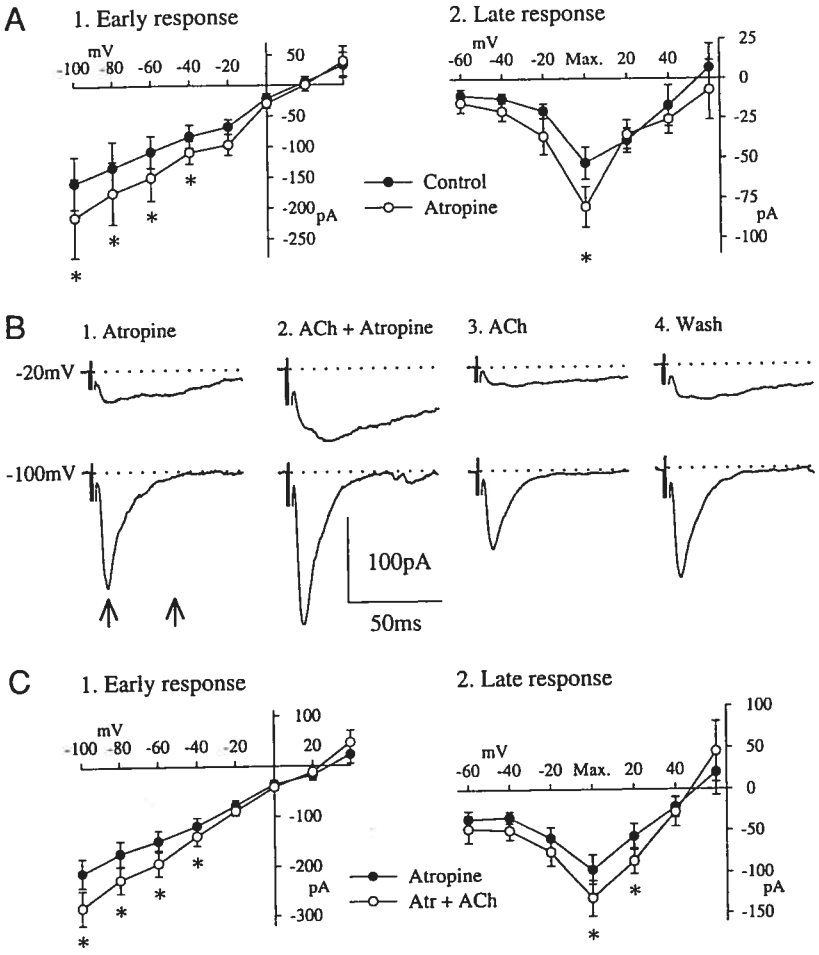


FIG. 4. Effects of the muscarinic receptor antagonist atropine on ACh inhibition of the EPSC. *A*: average $I_R$-$V_m$ of the early ($n = 5$; *1*) and late ($n = 5$; 2) components of the EPSC recorded before and during superfusion with atropine (10 $\mu$M). Asterisk, statistically significant difference between control and atropine treatment at this holding membrane potential (Student's *t*-test, $P < 0.05$). The increases produced by atropine ranged from 35 to 46% on the early response and were of 54% on the late response. *B*: current traces of the response evoked by single local electrical stimulus recorded at holding membrane potentials of −20 and −100 mV during superfusion with atropine (10 $\mu$M; *1*), with atropine and ACh (100 $\mu$M; 2), with ACh following washout of atropine (*3*) and following washout of ACh (*4*). Current traces represent the average of 8 sweeps and BMI (10 $\mu$M) was present in the superfusing medium throughout recording. Recordings were obtained from a MS neuron in a preparation from a P5 animal. Left and right vertical arrows in *B1* indicate where the early and late responses were measured, respectively. *C*: average $I_R$-$V_m$ of the early ($n = 13$; *1*) and late ($n = 8$; 2) components of the EPSC recorded during superfusion with atropine and atropine with ACh. In 5 neurons, the EPSC consisted only of an early component and these were included in the average of the early component. The $I_R$-$V_m$ of the late response was aligned on the holding membrane potential at which the response was maximum before averaging (usually at −20 or −40 mV). Asterisk, a statistically significant difference between atropine and atropine with ACh treatment at this holding membrane potential (Student's *t*-test, $P < 0.05$).

rons, whereas ACh in the presence of atropine produced no significant change in the remaining 5 (Fig. 4*C*). Similarly, the late component of the response was increased by an average 36% (at the membrane potential at which the response was largest) in eight of nine neurons tested, with no significant change observed in the remaining cell. Because ACh alone reduced the amplitude of evoked EPSCs while producing an increase when given in combination with atropine, we concluded that muscarinic receptor activation mediated inhibitory effects that masked an excitation possibly mediated by nicotinic receptors.

To identify the pharmacological type of muscarinic receptors mediating the inhibitory effects of cholinergic agonists, we tested the effect of CCh in the presence of the $M_1/M_4$ receptor antagonist pirenzepine. In three of four cases, CCh applied in the presence of pirenzepine (10 $\mu$M) produced no effect on the early component of the EPSC (110 ± 10%) while it produced an increase of the early response of 70% in the remaining case. Following washout of pirenzepine (103 ± 12% of control), CCh alone produced a significant decrease in all four cells relative to control (−33 ± 5%). CCh in the presence of pirenzepine produced similar effects on the late response: an increase was observed in two cells (24 and 31%, respectively), but no significant change in a third one. Following washout of pirenzepine (111 ± 14% of control), CCh alone produced a decrease in the late response of 36–53% in the three neurons tested. In conclusion, pirenzepine appeared to mimic the antagonistic effects of atropine, suggesting that the $M_1/M_4$ receptor mediated much of the inhibitory effects of cholinergic agonists.

### *Effects of the nicotinic receptor agonist*

To corroborate the existence of nicotinic receptor-mediated modulation of excitatory neurotransmission in the nAcb, we tested the specific nicotinic agonist DMPP (10 $\mu$M) with atropine (10 $\mu$M) present in the bathing medium throughout the experiments. DMPP was tested on both the composite EPSC ($n = 9$) and pharmacologically isolated AMPA/KA ($n = 7$) and NMDA ($n = 4$) receptor-mediated responses. Data from the two types of experiments were combined because similar results were obtained. As shown in a characteristic example in Fig. 5*A*, DMPP produced an enhancement of the EPSC that was similar to the one observed with ACh and CCh administered in the presence of atropine. DMPP increased the amplitude of the early AMPA/KA component of the EPSC in 13 of the 16 neurons (81%) tested and that of the late component in 12 of 13 neurons (92%) by an average of 37 ± 4% (19–80%) and 59 ± 8% (18–107%), respectively. Results are summarized in Fig. 5*B*. Statistically significant effects were observed at membrane potentials below −40 mV for the early response and only at the membrane potential at which the late response was maximal for the late responses. The effect of DMPP was statistically larger on the maximum of the late component of

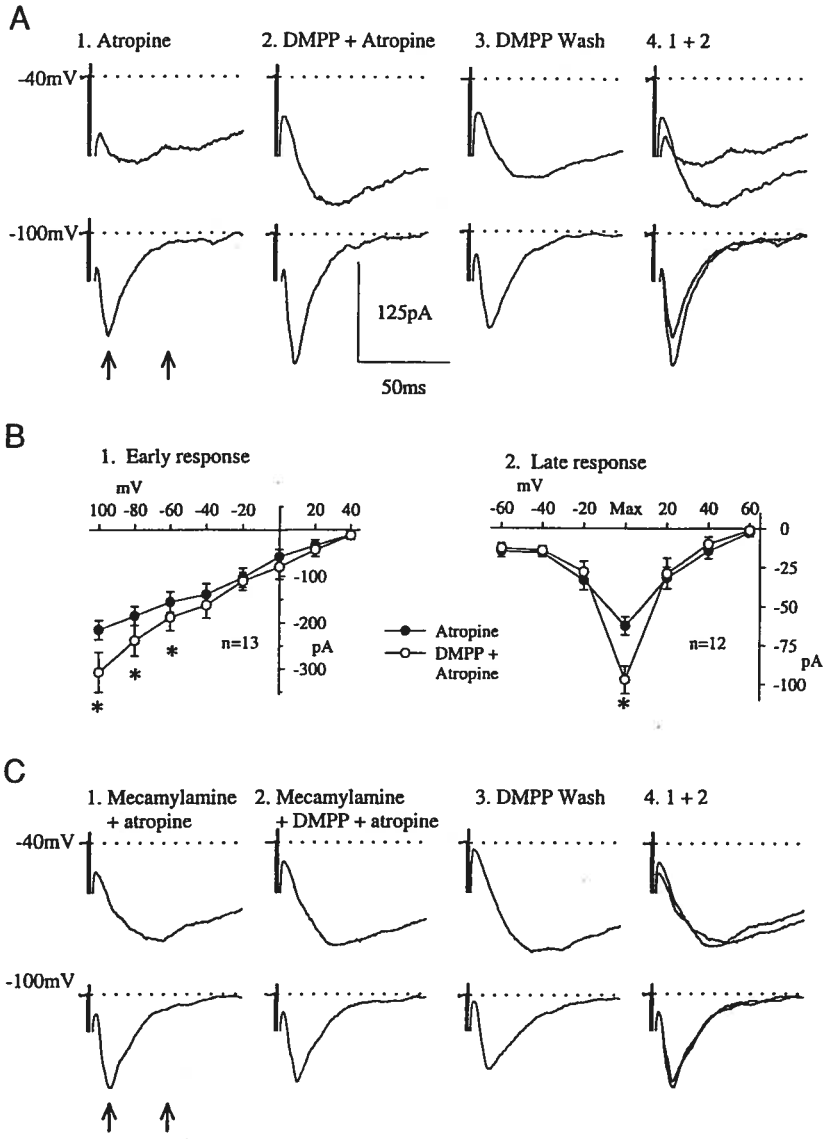


FIG. 5. Effects of nicotinic agonist and antagonist on the early and late EPSCs in the presence of atropine. *A*: current traces of the response evoked by single local electrical stimulus recorded at holding membrane potentials of −40 and −100 mV before (*1*), during (*2*), and after (*3*) superfusion with the nicotinic receptor agonist 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP; 10 μM). *4*: the overlay of the responses before and during DMPP application. Current traces represent the average of 8 sweeps and BMI (10 μM) and atropine (10 μM) were present in the superfusing medium throughout recording. Recordings were obtained from a MS neuron in a preparation from a P14 animal. Left and right vertical arrows in *1* indicate where the early and late responses were measured, respectively. *B*: average $I_R$-$V_m$ of the early ($n$ = 13; *1*) and late ($n$ = 12; *2*) components of the EPSC in which DMPP produced an increase of the response and recorded before and during superfusion with DMPP. The $I_R$-$V_m$ of the late response was aligned on the holding membrane potential at which the response was maximum (usually at −20 or −40 mV) before averaging. In 1 neuron, the EPSC consisted only of an early component and it was included in the average of the early component. The $I_R$-$V_m$ of the late response was aligned on the holding membrane potential at which the response was maximum before averaging (usually at −20 or −40 mV). Asterisk, a statistically significant difference between atropine and DMPP + atropine treatment at this holding membrane potential (Student's $t$-test, $P < 0.05$). *C*: current traces of the response evoked by single local electrical stimulus recorded at holding membrane potentials of −40 and −100 mV during superfusion with mecamylamine (*1*), mecamylamine and DMPP (*2*), and after washout of DMPP (*3*). *4*: the overlay of the responses during mecamylamine and mecamylamine with DMPP application. Recordings were obtained from a MS neuron in a preparation from a P6 animal. Other conventions are the same as in *A*. The scale bars in *A* also apply to *C*.

the EPSC than on the early one recorded at a holding membrane potential of −100 mV ($t_s$ = 2.423, df = 23, $P$ = 0.024). DMPP was always administered in the presence of atropine, showing that the enhancements were independent of muscarinic mechanisms. In addition, DMPP produced no changes in either the early or late components of the EPSC in four neurons when administered in the presence of the nicotinic receptor antagonist mecamylamine (Fig. 5*C*).

### *Effects of ACh as function of postnatal age*

We have recorded neurons from P1 to P27 animals, but most cells were recorded in preparations from P5 to P15 animals, and only with ACh we recorded a significant number of neurons over a range of postnatal ages sufficient to perform a developmental analysis (P3–P15, $n$ = 33). The magnitude of the inhibition produced by ACh as a function of postnatal age is presented in Fig. 6 for both the early and late responses. The effects of ACh on the early component of the EPSC did not change with postnatal age, but those on the late component increased significantly during the first two postnatal weeks. In the same group of neurons, we found no statistically significant changes in the amplitude of either the early ($r$ = 0.135, df = 26, $P$ = 0.492) or late ($r$ = 0.073, df = 24, $P$ = 0.721) component of the EPSC.

### *Locus of the cholinergic modulations of evoked EPSCs*

To identify the locus (pre- or postsynaptic) of action of cholinergic agonists, we compared several features of our recordings in the presence and absence of cholinergic agonists. Our evidence suggests that the effects produced by both muscarinic and nicotinic agonists were exclusively mediated by presynaptic mechanisms in the present study. First, we observed that ACh, DMPP, or ACh in the presence of atropine did not consistently produced changes in the holding membrane current at holding membrane potential between −100 and +40 mV (Fig. 7), suggesting that cholinergic agonists produced no change in input conductance. Similar results were obtained using steady-state current-voltage curves generated by slow voltage ramps between −100 and +40 mV (not shown). Second, ACh produced no change in the decay time

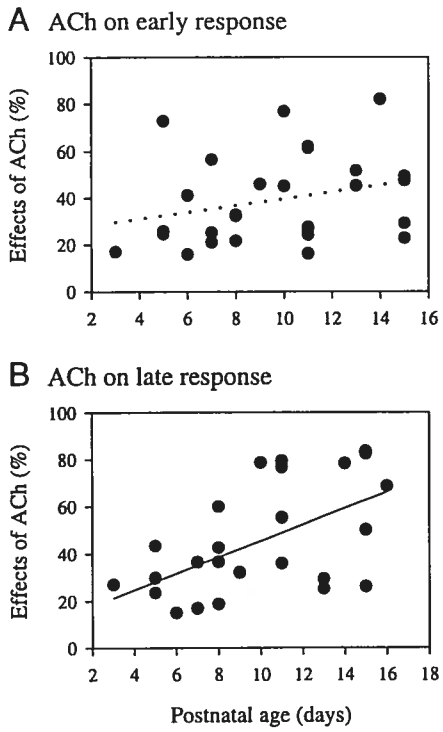


FIG. 6. Effects of ACh as a function of postnatal age. *A*: magnitude of the inhibitory effects of ACh on the early component of the EPSC recorded at a holding membrane potential of −100 mV as a function of postnatal age ($n$ = 28). No statistically significant correlation was found between the magnitude of the effects of ACh and postnatal age ($r$ = 0.265, df = 26, $P$ = 0.172; ···). *B*: magnitude of the inhibitory effects of ACh on the late component of the EPSC at the holding membrane potential at which the response was maximal as a function of postnatal age ($n$ = 26). A statistically significant correlation was found between the magnitude of the effects of ACh and postnatal age ($r$ = 0.556, df =24, $P$ = 0.003; —). ●, the effect observed for individual neuron in *A* and *B*.

($\tau$) of the evoked EPSC measured by fitting a single exponential to the isolated AMPA response (i.e., recorded in the presence of APV) at holding membrane potential of −100 mV (12.2 ± 1.4 and 13.2 ± 1.9 ms during control and during ACh administration, respectively, $n$ = 7, $P$ > 0.4) or −40 mV (12.6 ± 1.3 and 13.3 ± 1.6 ms during control and during ACh administration, respectively, $n$ = 7, $P$ > 0.7). Third, we used a paired-pulse protocol with a 50-ms interval between stimuli to discriminate between pre- and postsynaptic mechanisms (d'Alcatara et al. 2001; Hoffman and Lupica 2001; Mulder et al. 1996, 1997; Pennartz et al. 1991; Robbe et al. 2001; Zucker 1989). We found that the ratio (PPR; 2nd EPSC amplitude/1st EPSC amplitude) significantly changed during administration of agonists, thus suggesting presynaptic mechanisms. In the presence of ACh ($n$ = 7), the amplitude of the first and second evoked EPSCs both decreased, but the second response decreased to a larger extent, resulting in a decrease in the PPR (Fig. 8*A*). In contrast, during the application of DMPP ($n$ = 6), the amplitude of evoked EPSCs increased but the second response increased more, resulting in an increase in the PPR (Fig. 8*B*). Fourth, we found that ACh produced no effects on the response evoked by pressure ejection of glutamate in the vicinity of neurons in the presence of tetrodotoxin (TTX; Fig. 8*C*). Together, these results suggest that under the present experimental conditions muscarinic and nicotinic receptors agonists produced no detectable postsynaptic effects in the nAcb and that the present results reflect an action on presynaptic receptors.

Several studies in the nAcb and dorsal striatum have described direct postsynaptic effects on the passive and/or active membrane properties of MS neurons mediated by muscarinic receptors (Gabel and Nisenbaum 1999; Galarraga et al. 1999; Hsu et al. 1996, 1997; Pineda et al. 1995; Sugita et al. 1991; Uchimura and North 1990; see also Pennartz and Lopes da Silva 1994) consistent with the distribution of muscarinic receptor (Bernard et al. 1992; Weiner et al. 1990; Yan and Surmeier 1996). Several factors could explain the discrepancies between these studies and the present results, including the fact that we used whole cell patch clamp recording and that experiments were performed at room temperature. We found that under the present experimental conditions, cholinergic agonists produced a direct effect on the membrane and/or firing properties of MS neurons when QX-314 was omitted from the pipette solution. Figure 8*D* shows an example of the effects produced by CCh (50 μM) under these conditions. In this case, CCh produced a membrane depolarization of 21 mV. Intracellular depolarizing current pulse that was subthreshold during control readily evoked spiking when CCh was added to the bath, and the number of action potentials increased in response to suprathreshold current injection. These results suggest the that the presence of QX-314 into the recording pipette occluded the postsynaptic effects mediated by muscarinic receptors.

## DISCUSSION

We have studied the effects of cholinergic agonists on isolated EPSCs in nAcb MS neurons. Broad-spectrum cholinergic

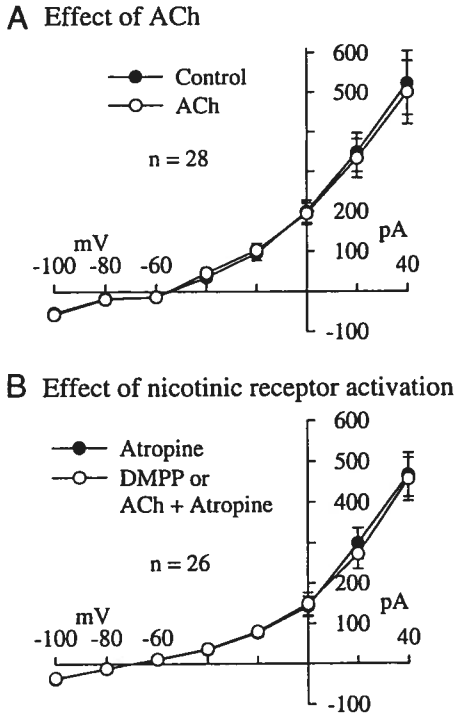


FIG. 7. Effects of cholinergic agonists on holding membrane currents. *A*: average $I_{hold}$-$V_m$ before and during superfusion with ACh (100 μM). *B*: same as *A* but during superfusion of atropine and during superfusion with DMPP or ACh in the presence of atropine. No statistically significant differences were found at any holding membrane potentials.

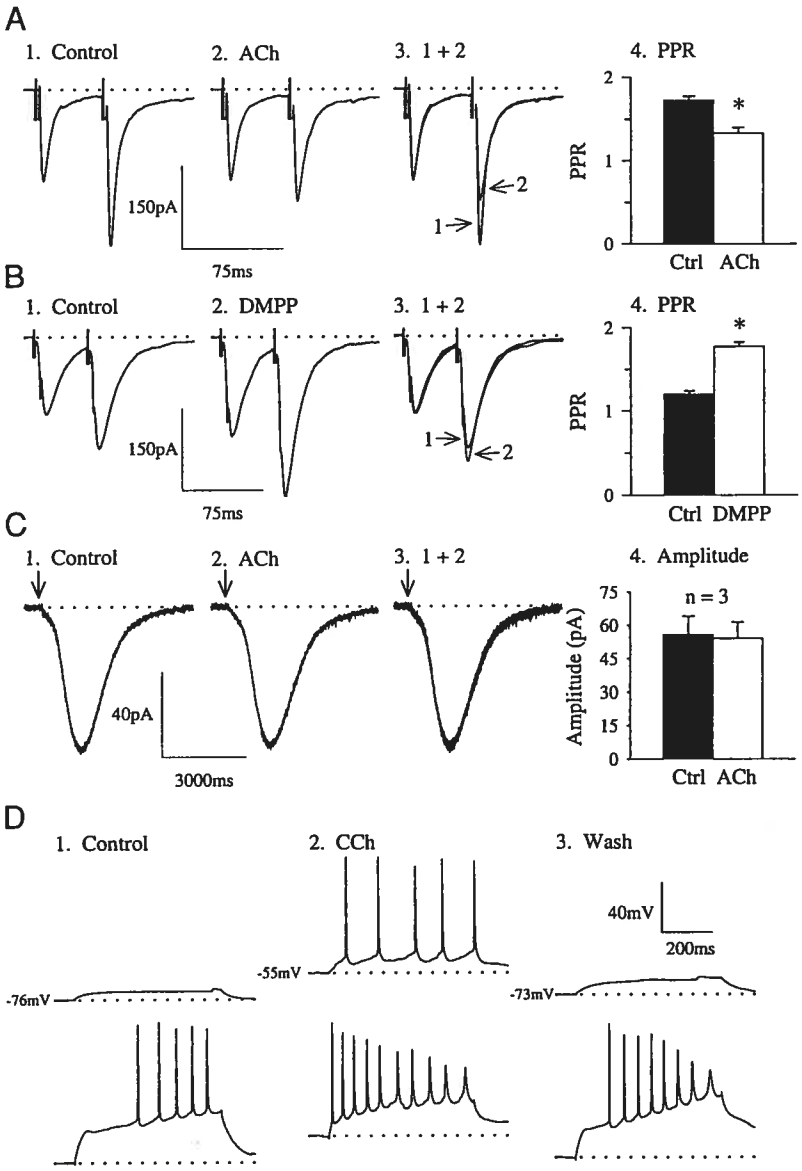


FIG. 8. Locus of the effects of cholinergic agonist on EPSCs. *A*: effects of ACh on PPR. Current traces of the responses evoked by a pair of single local electrical stimuli 50 ms apart at holding membrane potentials of −100 mV before (*1*) and during (*2*) superfusion with ACh (100 μM). *3*: the overlay of the responses before and during ACh application; the amplitude of the 1st response in the presence of ACh was scaled to match the amplitude of the 1st response during control. Note that the 2nd response proportionally decreased more than the 1st response in the presence of ACh and that there is no apparent changes in the time course of the EPSCs. Current traces represent the average of 8 sweeps and BMI (10 μM) was present in the superfusing medium throughout recording. *4*: the average amplitude of the PPR from 7 neurons before and during superfusion with ACh. PPR was statistically larger in the presence of ACh than during control condition ($t_s$ = 3.045, df = 6, $P$ = 0.023). *B*: effects of DMPP on PPR. Conventions are the same as in *A*. Note that the 2nd response proportionally increased more than the 1st response in the presence of DMPP and that there is no apparent changes in the time course of the EPSCs. PPR was statistically larger in the presence of DMPP than during control condition ($t_s$ = 3.660, df = 5, $P$ = 0.015). *C*: current traces of the response evoked by local pressure ejection of glutamate (1 mM) from a patch pipette before (*1*) and during (*2*) superfusion with ACh (100 μM) at a holding membrane potential of −100 mV in the presence of tetrodotoxin (1 μM) and BMI (10 μM). *3*: the overlay of the responses recorded in *1* and *2*. *4*: the amplitude of the peak response recorded at a holding membrane potential of −100 mV for 3 neurons before and during superfusion with ACh. *D*: effect of the cholinergic agonist carbachol on the membrane and firing properties of MS neurons. Voltage responses evoked with intracellular current pulses of 15 (*top*) and 75 pA (*bottom*) from resting membrane potential before (*1*), during (*2*), and after (*3*) the addition of carbachol (50 μM) to the superfusing medium. In this case, the addition of carbachol produced a 21-mV depolarization of the membrane potential. Recordings were obtained under the same experimental conditions as the other neurons in the present study with the exception that QX-314 was omitted from the pipette solution.

agonists ACh and CCh produced a reduction of both AMPA/KA and NMDA receptor-mediated components of the EPSC. In contrast, in the presence of the muscarinic receptor antagonist atropine, cholinergic agonists produced an increase of the EPSC, suggesting that the inhibition of the EPSC was mediated by muscarinic cholinergic receptors and that, under the present experimental conditions, an excitatory effect mediated by nicotinic cholinergic receptors was masked by muscarinic-mediated inhibition. The effects of atropine were generally mimicked by the antagonist pirenzepine, suggesting that the inhibitory effects of cholinergic agonists were mediated in part by $M_1/M_4$ type of muscarinic receptor. DMPP, a specific nicotinic receptor agonist, produced an enhancement of the EPSC. However, this effect was blocked by mecamylamine, demonstrating the presence of a modulation of the EPSC mediated by nicotinic receptors. Cholinergic agonists apparently produced no postsynaptic effects, but produced consistent changes in the paired-pulse ratio. Conversely, they produced no effects on responses evoked by brief glutamate ejection in the vicinity of the recorded neurons in the presence of TTX, showing that the cholinergic agonists were acting on presynaptic receptors, probably located on glutamatergic terminals. Because cholinergic agonists produced direct effects on the membrane and firing properties of MS neurons when QX-314 was omitted from the pipette recording solution, intracellular QX-314 could constitute an interesting pharmacological tools for studying presynaptic mechanisms. Together, these results suggest that ACh modulates glutamatergic neurotransmission by decreasing glutamate release via an action on presynaptic muscarinic receptors or by increasing glutamate release via nicotinic receptors. These contrasting effects of ACh on single neurons emphasizes the complexity of cholinergic modulation of glutamatergic neurotransmission in the nAcb. In addition to its presynaptic effects on glutamatergic neurotransmission, ACh produces a direct modulation of the membrane and firing properties of MS neurons and is also known to modulate the release of other neurotransmitters in the nAcb. We suggest that ACh may play an important role in the nAcb by gating glutamatergic excitation. This function may be important for synapse formation and consolidation during postnatal develop-

ment as well as in controlling MS neurons membrane bistability in mature nAcb.

*Locus of cholinergic receptors*

Our results suggest that both muscarinic depression and nicotinic potentiation of EPSCs were mediated by an action on cholinergic receptors located on glutamatergic terminals. We found that neither CCh and ACh or DMPP altered the input conductance nor changed the time course (the rise or decay phase) of EPSCs. Cholinergic agonists also produced consistent changes in the paired-pulse ratio in agreement with an action mediated by presynaptic mechanisms (d'Alcatara et al. 2001; Hoffman and Lupica 2001; Mulder et al. 1996, 1997; Pennartz et al. 1991; Robbe et al. 2001; Zucker 1989). Furthermore, ACh produced no effects on the response evoked by exogenous glutamate during blockade of synaptic transmission with TTX. In contrast, CCh produced marked effects on the membrane and firing properties of MS neurons when QX-314 was omitted from the pipette recording solution. We conclude that the modulation of EPSCs by cholinergic agonists was mediated by presynaptic mechanisms. Our findings are in general agreement with studies on the effects of muscarinic agonists on glutamatergic neurotransmission in both the nAcb (Pennartz and Lopes da Silva 1994; Sugita et al. 1991) and the dorsal striatum (Akaike et al. 1988; Barral et al. 1999; Hernandez-Echeagaray et al. 1998; Hsu et al. 1995; Malenka and Kocsis 1988), whereas, to our knowledge, there have been no reports on the modulation of glutamatergic neurotransmission by nicotinic receptor in either structure.

In agreement with Pennartz and Lopes da Silva (1994), we observed no changes in the passive membrane properties of MS neurons in the presence of cholinergic agonists, whereas several studies on the nAcb and dorsal striatum have described direct postsynaptic effects on the passive and/or active membrane properties of MS neurons mediated by muscarinic receptors. These observations suggest that postsynaptic activation of muscarinic receptors enhanced the excitability of MS neurons by producing membrane depolarization (Hsu et al. 1996; Sugita et al. 1991; Uchimura and North 1990) and an increase in input resistance (Galarraga et al. 1999; Hsu et al. 1996; Pineda et al. 1995; Uchimura and North 1990) likely by reducing $K^+$ conductances including inward rectifying ($I_{Kr}$) and persistent ($I_{Krp}$) (Gabel and Nisenbaum 1999; Galarraga et al. 1999; Hsu et al. 1996, 1997; Pineda et al. 1995). These results are consistent with the membrane potential depolarization we observed when QX-314 was omitted from the pipette solution that likely resulted from the suppression of these $K^+$ conductances. Postsynaptic effects mediated by muscarinic receptors were typically blocked by pirenzepine and attributed to the activation of $M_1$ receptors. These observations are consistent with the distribution of muscarinic receptors in the nAcb and striatum where $M_1$ receptors are primarily found postsynaptically on MS neurons (Bernard et al. 1992; Weiner et al. 1990; Yan and Surmeier 1996).

Because muscarinic receptors are coupled to G protein (Caulfield and Birdsall 1998), one possibility is that by using a whole cell recording technique, we washed out some elements of the second-messenger system necessary for the expression of postsynaptic effects even though ATP and GTP were always included in the pipette solution. Alternatively, we routinely added QX-314 to the recording pipette solution to block action potential generation. We found that by omitting QX-314 from the pipette recording solution, cholinergic agonists modulated the membrane and firing properties of MS neurons, suggesting that QX-314 occluded the postsynaptic effects of cholinergic agonists. In addition to blocking voltage-gated $Na^+$ channels, QX-314 is also known to inhibit G-protein-gated $K^+$ conductances (Alreja and Aghajanian 1994; Andrade 1991; Lambert and Wilson 1993; Nathan et al. 1990; Otis et al. 1993; Slesinger 2001) and may have occluded muscarinic postsynaptic effects on $K^+$ conductances (Gabel and Nisenbaum 1999; Galarraga et al. 1999; Hsu et al. 1996, 1997; Pineda et al. 1995). This hypothesis is consistent with recent findings showing that intracellular QX-314 blocks muscarinic $M_1$ and $M_3$ receptor signaling pathways expressed in *Xenopus* oocytes (Hollmann et al. 2000, 2001). The present results suggest that internal QX-314 may also block the signaling pathway of native muscarinic receptors and that it could be a useful pharmacological tool to isolate presynaptic mechanisms in the study of the muscarinic cholinergic system or other neurotransmitter systems modulating G-protein-gated $K^+$ conductances. Further studies would be needed to test these hypothesis.

To our knowledge, this is the first study reporting a modulation of glutamatergic neurotransmission mediated by nicotinic receptors in the nAcb or other neostriatal structures. Some of the previous studies on the nAcb and dorsal striatum have limited their scope to muscarinic receptor-mediated modulation of excitatory neurotransmission (Barral et al. 1999; Calabresi et al. 1998; Hernandez-Echeagaray et al. 1998; Sugita et al. 1991). In studies in which general cholinergic agonists were used, none reported an increase in excitatory neurotransmission in the presence of muscarinic antagonists (Hsu et al. 1995; Pennartz and Lopes da Silva 1994), whereas Akaike et al. (1988) found that nicotine produced no effect on excitatory postsynaptic potentials (EPSPs) in the caudate nucleus of adult rats.

The presence of functional presynaptic nicotinic receptors in the nAcb and dorsal striatum has been documented (see Lendvai and Vizi 1999; MacDermott et al. 1999). Recent studies in other regions of the CNS have found that nicotinic agonists potentiated glutamatergic neurotransmission presumably by acting on presynaptic receptors located on glutamatergic terminals (Aramakis and Metherate 1998; Gil et al. 1997; Girod et al. 2000; Gray et al. 1996; Radcliffe and Dani 1998; McGehee et al. 1995; Vidal and Changeux 1993) in agreement with the present findings. Nicotine has also been found to exert direct postsynaptic excitation on some specific neuronal populations, including interneurons in the cerebral cortex (McCormick and Prince 1986; Porter et al. 1999; Roerig et al. 1997) and hippocampus (Frazier et al. 1998; Jones and Yakel 1997; McQuiston and Madison 1999), dopaminergic neurons in the ventral tegmental area (Calabresi et al. 1989; Pidoplichko et al. 1997), retinal ganglion cells (Feller et al. 1996), and in brain stem nucleus ambiguus (Zhang et al. 1993), but we found no evidence for a similar action in nAcb MS neurons. Nicotinic receptors are ligand-gated channels independent of second-messenger system and would not be occluded by QX-314 in the same way as muscarinic receptors.

*Muscarinic depression of EPSCs*

Few studies have examined the modulatory role of ACh on glutamatergic neurotransmission in the nAcb. Pennartz and Lopes da Silva (1994) reported that in ventral striatal slices muscarine and CCh reversibly attenuated the EPSP through presynaptic mechanisms and that this action was completely antagonized by atropine or pirenzepine in agreement with our findings. They also found that increasing endogenous levels of ACh with acetylcholinesterase inhibitors resulted in a decrease in the EPSP in accordance with our finding that endogenous ACh exerted a tonic depression of EPSC, as suggested by the increase in the EPSC produced by atropine alone in our preparation. Sugita et al. (1991) also reported that cholinergic muscarinic receptor activation depressed glutamatergic neurotransmission in the nAcb through presynaptic mechanisms.

Comparable results have been obtained in the dorsal striatum, a structure that shares several anatomical and physiological characteristics with the nAcb and in which cholinergic and muscarinic agonists have been found to decrease the responsiveness of MS neurons to excitatory inputs, presumably by acting on presynaptic muscarinic receptors (Akaike et al. 1988; Barral et al. 1999; Hernandez-Echeagaray et al. 1998; Hsu et al. 1995; Malenka and Kocsis 1988). Therefore our findings that muscarinic receptors depressed glutamatergic EPSCs by acting on presynaptic receptors are in general agreement with previous studies.

Previous studies on the nAcb and dorsal striatum made no attempt to examine the possibility that cholinergic agonists exerted differential modulation of AMPA/KA and NMDA receptor-mediated excitation. We found that the activation of muscarinic receptors depressed both AMPA/KA- and NMDA-mediated EPSCs and that with CCh the depression was larger on the NMDA than on the AMPA/KA receptor-mediated component. The larger depression of the NMDA-mediated response could be the result of rundown of the NMDA response in vitro, but the fact that we observed a larger increase in the NMDA receptor-mediated component than on the AMPA/KA-mediated response with nicotinic receptor agonists suggests that this was not the case. Alternatively, it is possible that the effects are mediated by different types of muscarinic receptors for which ACh and CCh have different binding characteristics.

There are no highly selective antagonists for muscarinic receptor subtypes (Caulfield and Birdsall 1998), and we did not perform extensive pharmacological studies to identify the subtype of muscarinic receptor involved in the inhibition of the EPSC. We found that pirenzepine, which acts predominantly on $M_1$ and $M_4$ receptors, mimicked much of the effects of atropine. These results are in agreement with those of Pennartz and Lopes da Silva (1994). Others have suggested that muscarinic receptor-mediated inhibition in the nAcb and dorsal striatum were mediated by $M_3$ (Hsu et al. 1995; Sugita et al. 1991) or $M_2$-$M_3$ (Hernandez-Echeagaray et al. 1998) receptors. A subset of $M_1$, $M_3$, and $M_4$ muscarinic receptors are found on axon terminals forming asymmetrical synapses (Hersch and Levey 1995; Hersch et al. 1994) and provide an anatomical basis for the presynaptic modulation of glutamatergic neurotransmission by ACh. In contrast, the $M_2$ receptor appears to be located on axon terminals making symmetrical synapses, suggesting that they do not participate in the modulation of excitatory input. $M_1$ and $M_3$ receptors mRNA are found in cortical and hippocampal pyramidal neurons as well as in the amygdala and thalamus (Buckley et al. 1988; Wei et al. 1994), and these structures could be the source of presynaptic muscarinic receptors located on glutamatergic terminals in the nAcb.

*Nicotinic potentiation of EPSCs*

ACh and CCh not only act on muscarinic receptors but also activate nicotinic receptors. Under the present experimental conditions, nicotinic receptor-mediated excitation became apparent only when appropriate muscarinic receptor antagonists were added to the superfusing medium, suggesting that nicotinic receptor-mediated excitation was masked by a predominant muscarinic inhibition. Furthermore, the application of DMPP mimicked the enhancing effects produced by general cholinergic agonists in the presence of atropine or pirenzepine, and this effect was blocked by mecamylamine, a specific nicotinic receptor antagonist, showing that the potentiation of the EPSC was mediated by the activation of nicotinic receptors. To our knowledge, this is the first demonstration that glutamatergic neurotransmission is modulated by nicotinic presynaptic receptors in the nAcb.

The presence of presynaptic nicotinic cholinergic receptors has been documented in both the nAcb and dorsal striatum (see Lendvai and Vizi 1999; MacDermott et al. 1999). Our findings are in agreement with several recent studies showing that the activation of presynaptic nicotinic cholinergic receptors facilitates glutamatergic neurotransmission in different regions of the CNS (e.g., Aramakis and Metherate 1998; Girod et al. 2000; Gray et al. 1996; Guo et al. 1998; McGehee et al. 1995). These studies suggested that facilitation of glutamatergic neurotransmission was mediated by nicotinic receptors containing the $\alpha$ subunit. Our results suggest that another type of nicotinic receptor is involved in the nucleus accumbens because receptors containing the $\alpha$ subunit are insensitive to mecamylamine (e.g., MacDermott et al. 1999). Our results are supported by recent findings showing that nicotine increases glutamate release in the nAcb via a mecamylamine-sensitive nicotinic receptor (Reid et al. 2000).

Several studies have demonstrated that local nicotinic receptor activation increased dopamine release in the nAcb (Fu et al. 2000; Hildebrand and Svensson 2000; Nisell et al. 1994a,b) raising the possibility that some of the effects we observed were indirectly mediated through the dopaminergic system. This appears unlikely because nicotinic-evoked dopamine release in the nAcb has been found to be insensitive to mecamylamine but is sensitive to $\alpha$ subunit antagonists (Fu et al. 2000), suggesting that a different type of nicotinic receptors control glutamate and dopamine release in the nAcb.

We have found that nicotinic agonist enhanced both APMA/KA and NMDA receptor-mediated EPSCs but that the effect was statistically larger on NMDA- than on AMPA/KA-mediated response. This is in partial agreement with Aramakis and Metherate (1998), who found that in rat auditory cortex during postnatal development nicotine selectively enhanced NMDA receptor-mediated EPSP while producing no change in AMPA/KA receptor-mediated EPSP. The authors concluded that nicotinic receptors were located on glutamatergic terminals at synapses containing only NMDA receptors, whereas the present results suggest that nicotinic receptors are located on

terminals containing both AMPA/KA and NMDA receptors, whereas a subclass contains only NMDA receptors.

Aramakis and Metherate (1998) reported that nicotinic modulation of NMDA receptor-mediated EPSC was only observed in preparations from animals less than 19 days old. In the present study, we used animals of an age comparable to those used by these authors, whereas previous studies on the nAcb and dorsal striatum used adult animals. This raises the possibility that nicotinic modulation of glutamatergic transmission is also developmentally regulated in the nAcb. Indeed, the expression of different nicotinic receptor subunit mRNA appears to be developmentally regulated in the nAcb as well as in brain regions providing glutamatergic innervation to the nAcb (Aubert et al. 1996; Cimino et al. 1995; Fiedler et al. 1990; Hellstrom-Lindahl et al. 1998; Shacka and Robinson 1998; Zhang et al. 1998), suggesting that nicotinic modulation may vary with the stage of development. We found that the inhibitory effects of ACh increased during the first two postnatal weeks. This developmental change could result from an increase in the number of muscarinic receptors on glutamatergic terminals or, alternatively, in a decrease in nicotinic receptors. Further experiments are needed to explore these possibilities.

### *Functional considerations*

The nAcb constitutes an important point of convergence of information from several limbic structures, including the prefrontal cortex (PFC), the amygdala, the hippocampus and midline thalamic nuclei (Groenewegen et al. 1980, 1982, 1987; Jayaraman 1985; Kelley and Domesick 1982; Kelley and Stinus 1984; Kelley et al. 1982; Krayniak et al. 1981; Newman and Winans 1980). These afferent systems, which are believed to be glutamatergic, are thought to mediate their excitatory drive mainly through AMPA/KA and NMDA glutamatergic receptors (DeFrance et al. 1985; Finch 1996; Kombian and Malenka 1994; Nicola et al. 1996; Yim and Mogenson 1982; Zhang and Warren 1999). Because we used local electrical stimulation, the EPSCs recorded in the present study were probably evoked by the activation of these pathways. Our results as well as these of others show that ACh exerts complex control over the excitability of nAcb MS neurons by acting at both pre- and postsynaptic levels. At presynaptic level, we found that ACh can increase or decrease the efficacy of incoming glutamatergic input possibly by controlling glutamate release through an action on nicotinic and muscarinic receptors, respectively. Postsynaptically, ACh increases the excitability and responsiveness of MS neurons by acting on postsynaptic muscarinic receptors located on MS neurons.

We studied the effects of cholinergic agonists during postnatal development between P1 and P27. During that period, the intrinsic and firing properties of nAcb MS neurons mature and appear to become adult-like only by the end of the third postnatal week (Belleau and Warren 2000). In addition, during the first 10 postnatal days, nAcb MS neurons are essentially aspiny and possess varicose dendrites, whereas they assume an adult spiny appearance only toward the end of the third postnatal week (unpublished observation). Similar developmental changes were found in MS neurons in the developing dorsal striatum and were accompanied by a large increase in the density of excitatory synapses, particularly on spines (Sharpe and Tepper 1998; Tepper and Trent 1993; Tepper et al. 1998). Presumably, excitatory synapse formation and consolidation is also taking place in the nAcb during the postnatal period. During postnatal development, behavioral experience is thought to shape and refine neural circuits through activity-dependent mechanisms (Aamodt and Constantine-Paton 1999; Collingridge and Singer 1990; Fox et al. 1999), and the disruption of both glutamatergic and cholinergic functions has been shown to reduce developmental plasticity in some regions of the neuraxis (Aramakis et al. 2000; Bear and Singer 1986; Bear et al. 1988, 1990; Brooks et al. 1997; Cantallops and Routtenberg 1999; Iwasato et al. 2000). In adult animals, the normal function of MS neurons involves the interactions between their intrinsic properties and their glutamatergic inputs, whereas different glutamatergic inputs from different sources appear to have different functions in initiating MS neuron activation (O'Donnell and Grace 1995). Part of this organization could be triggered by activity-dependent mechanisms involving glutamatergic neurotransmission, especially when mediated by NMDA-type receptors (Craig and Lichtman 2000). Indeed, functional glutamatergic innervation of MS neurons is already present on the day of birth and NMDA receptor-mediated EPSCs are preponderant during the first two postnatal weeks, whereas AMPA/KA receptor-mediated EPSCs predominate in juvenile and adult animals (Zhang and Warren 1999). The cholinergic modulation of glutamatergic neurotransmission during the postnatal period possibly contributes to the maturation and refinement of the glutamatergic innervation of the nAcb. In addition, glutamatergic innervation of the nAcb is topographically organized and ACh could participate in the refinement of this organization by turning on and off specific inputs in the nAcb.

Disruption of some of the glutamatergic inputs to the nAcb during early postnatal development (P7) has been found to produce enduring behavioral changes (Al Amin et al. 2001; Flores et al. 1996a,b; Lipska et al. 1993; Sams-Dodd et al. 1997; Weinberger and Lipska 1995; Wood et al. 1997; see also Lipska et al. 1998) as well as changes in dopaminergic receptors (Baca et al. 1998; Flores et al. 1996a,b) and dopamine release (Lillrank et al. 1999) in the nAcb. Typically, these changes are expressed only after puberty, and, interestingly, the same lesions at P14 or in adult animals produced no comparable changes (Wood et al. 1997), suggesting that there is a critical period during which developmental plasticity can be expressed in the nAcb.

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Present address of L. Zhang: Center for Neuroscience, University of California, 1544 Newton Ct., Davis, CA 95616.

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# CHAPTER IV

# Preferential inhibition of NMDA receptor-mediated EPSCs by dopamine during development in nucleus accumbens

Liming Zhang[1,2] and Richard A. Warren[1,3]

[1]Centre de recherche Fernand-Seguin, [2]Department of Physiology and [3]Department of Psychiatry

University of Montreal, Montreal, Canada

Running title: DOPAMINERGIC POSTNATAL REGULATION OF EPSCS IN NUCLEUS ACCUMBENS

Address for correspondence:

Richard A. Warren
Centre de recherche Fernand -Seguin
7331 Hochelaga street
Montreal, Quebec, Canada, H1N 3V2
Tel. (514) 251-4015 ext. 3415
Fax: (514) 251-2617
e.mail: richard.warren@umontreal.ca

## ABSTRACT

We studied the effects of dopamine (DA) on glutamatergic excitatory postsynaptic currents (EPSCs) of medium spiny (MS) neurons in nucleus accumbens (nAcb) during postnatal development using whole-cell patch-clamp technique *in vitro*. The EPSCs evoked in MS neurons by local nAcb stimuli displayed both early and late ionotropic glutamate receptor-mediated components that could be respectively abolished with specific AMPA/KA and NMDA receptors antagonists. The addition of DA to the supefusing medium produced a marked decrease of the evoked EPSCs. The AMPA/KA receptor-mediated response was on average abolished by 40% whereas the NMDA receptor-mediated component of the response was decreased by 91%, an effect comparable to the effect produced by specific NMDA receptor antagonist. The effects of DA on evoked EPSCs were mimicked by the $D_1$-like receptor agonist, SKF 38393 and were antagonized by the $D_1$-like receptor antagonist SCH 23390 but not by the $D_2$-like receptor antagonist sulpiride or by clozapine. DA did not change either the membrane input conductance nor the characteristics of PSCs evoked by the local administration of glutamate in the presence of tetrodotoxin. In contrast, DA altered the paired-pulse ratio of evoked EPSCs at a holding membrane potential of -100mV but not at -40mV. The action of DA was resistant to protein kinase inhibitors H89 and Ro-32-0432, suggesting that DA-induced EPSCs depression did not involve protein kinase A nor C. These results suggest that the inhibitory effects of DA on EPSCs are mediated by both pre and postsynaptic mechanisms in the nAcb. The present results show that the activation $D_1$-like dopaminergic receptors modulates glutamatergic neurotransmission by preferentially

abolishing NMDA receptor-mediated EPSCs by acting independently of PKA and PKC through both pre- and postsynaptic mechanisms.

**Key Words:** Nucleus accumbens; excitatory postsynaptic currents; DA modulation; NMDA and AMPA receptors; postnatal development.

## RÉSUMÉ

Les effets de la dopamine (DA) sur la transmission glutamatergique dans les neurones épineux moyens GABAergiques du noyau accumbens (nAcb) ont été examinés dans des tranches de rat en utilisant la technique *whole-cell patch-clamp* pendant le développement postnatal. Les CPSEs évoqués par une stimulation locale dans le nAcb comprenaient généralement deux composantes: une précoce médiée par des récepteurs AMPA/KA et une tardive médiée par des récepteurs NMDA. L'ajout de DA a produit une inhibition beaucoup plus importante de la réponse médiée par les récepteurs NMDA (-92%) que de celle médiée par les récepteurs AMPA/KA (-40%). Les effets de la DA sur les réponses NMDA étaient comparables à ceux produits par l'APV, un antagoniste spécifique des récepteurs NMDA. Les effets de la DA sur les CPSEs pouvaient être reproduits par un agoniste (SKF 38393 ) des récepteurs D1-semblables et bloqué par un antagoniste (SCH 23390) des mêmes récepteurs alors que les substances agissant sur des récepteurs D2-semblables ne produisaient aucun effet. La DA a changé le rapport des réponses à des stimuli pairés, mais n'avait aucun effet sur les propriétés membranaires des neurones étudiés ou sur les réponses évoquées par l'injection de glutamate en présence de tétrodotoxine. Ces résultats suggèrent que les effets de la DA étaient principalement médiés par des mécanismes présynaptiques pendant le développement postnatal. La dépression des CPSEs induite par la DA ne semble pas impliquer la protéine kinase A ni la protéine kinase C car elle était résistante aux inhibiteurs de protéines kinases H89 et Ro-32-0432. Ces résultats démontrent que l'activation des récepteurs dopaminergiques D1-semblables dans le nAcb diminue la libération de

glutamate et suppriment de préférence les CPSEs médiés par les récepteurs NMDA pendant une étape cruciale du développement postnatal.

**Mots-Clés** : noyau accumbens, courants postsynaptiques excitateurs, modulation DA, récepteur NMDA, développement postnatal

## INTRODUCTION

Nucleus accumbens (nAcb) which forms the ventral part of the striatum has been proposed to serve as an interface between the limbic system and the motor system (Mogenson et al., 1980; Groenewegen et al., 1996). While the nAcb receives a dense glutamatergic innervation from the prelimbic cortex and different limbic structures, including the hippocampus and amygdala (for review, see Zahm and Brog, 1992; Finch, 1996; Groenewegen et al., 1996; Heimer et al., 1997), it also receives a massive dopamine (DA) input from ventral tegmental area (VTA) of midbrain. An important feature of these glutamatergic and dopaminergic afferents to the nAcb is that they converge on the same dendritic spines of medium spiny (MS) GABAergic projecting neurons (Bouyer et al., 1984; Totterdell and Smith, 1989; Seaack and pickle, 1990; Johnson et al., 1994; Meredith, 1999). This closed spatial relationship suggests a possible interaction between the glutamatergic and dopaminergic systems at the pre- and/or postsynaptic levels. Behavioral studies have shown that interactions between DA and glutamatergic synaptic transmission, particularly those mediated by NMDA receptors, play a key role in animal behaviors associated with the nAcb (see Vanderschuren and Kalivas, 2000; Baldo et al., 2002). Recent finding of $D_1$/NMDA receptor complexes in striatal and hippocampal tissue indicates possible direct protein-protein interactions between $D_1$ and NMDA receptors (Lee et al., 2002).

In the nAcb, expression of the NMDA receptor-dependent form of long-term potentiation has been demonstrated (Pennartz et al., 1993; Kombian and Malenka, 1994; Schramm et al., 2002) and plasticity within nAcb is thought to mediate instrumental

learning processes and many aspects of drug addiction in which coincident activation of NMDA and dopamine $D_1$ receptors is required (Kelley et al., 1997; Smith-Roe and Kelley, 2000; Baldwin et al., 2000; Hernandez et al., 2002). The nAcb may thus constitute a locus where NMDA receptors promote drug reinforcement (Maldve et al., 2002). In addition to aspects of instrumental learning and drug addiction, the nAcb appears to be involved in a number of functions such as motivation, attention and reward (Mogenson et al., 1980; Kalivas and Nakmura, 1999) under the control of the mesolimbic dopaminergic system (Willner et al., 1991).

Despite the well-known role of nAcb DA in the modulation of motivated behaviors, and despite recent advances in the understanding of cellular and molecular aspects of dopaminergic and glutamatergic receptor interaction (Lee et al., 2002), the precise mechanisms underlying DA and NMDA receptors interaction to be reflected on EPSCs in the nAcb remain unclear. Some studies reported that the activation of $D_1$ receptors enhanced NMDA receptor-mediated EPSCs (NMDAR-EPSCs) in dorsal striatal slices (Cepeda et al., 1993; Cepeda and Levine, 1998; Levine et al., 1996a, b), while others reported that $D_1$ receptor agonists attenuated NMDAR-EPSCs in MS striatal neurons in culture (Lee et el., 2002; Lin et al., 2003). Additionally, certain investigators reported that DA or $D_1$ receptor agonists potentiate NMDAR-EPSCs in the nAcb slices (Harvey and Lacey, 1997; Chergui and Lacey, 1999), while others reported no significant modulatory effects of DA on NMDAR-EPSCs (Nicola and Malenka, 1998; Beurrier and Malenka, 2002). Although electrophysiological studies in brain slices have shown that activation of $D_1$ receptors inhibit glutamatergic synaptic transmission in the nAcb by a

presynaptic action (Higashi et al., 1989; Pennartz et al., 1992; Harvey and Lacey, 1996; Nicola et al., 1996; Nicola and Malenka, 1997, 1998; Beurrier and Malenka, 2002), the inhibitory effect of DA on EPSCs by presynaptic locus via the $D_1$ receptor was only determined on the compound current levels, in particular, on AMPA/KA receptor-mediated EPSCs (AMPA/KAR-EPSCs) in the nAcb. A substantive effect of DA on pharmacologically isolated NMDAR- and AMPA/KAR-EPSCs remains unknown.

In a previous study, we showed that ACh presynaptically modulated AMPA/KAR- and NMDAR-EPSCs in a parallel fashion in the nAcb (Zhang and Warren, 2002). In an effort to clarify how the NMDAR- and AMPA/KAR-EPSCs might be affected in the nAcb by dopaminergic innervation, we investigated the effect of DA on NMDAR and AMPA/KAR-excitatory synaptic transmission in this region. Our results demonstrate that DA depresses the excitatory input onto MS neurons probably by activation of pre- and postsynaptic $D_1$-like receptors. While DA depressed the elicited AMPA/KAR-EPSCs in MS neurons by 40% of control, evoked NMDAR-EPSCs were almost completely abolished.

## MATERIALS AND METHODS

***Slice preparation*** The procedure used for preparing slices has been previously described (Belleau and Warren, 2000; Zhang and Warren, 2002). One to 20-day-old (P1-P20) Sprague Dawley rat pups of either sex were used in the present experiments. P5 and younger pups were anaesthetized by hypothermia whereas P6 and older animals were anesthetized by inhalation of methoxyfluran vapor in a closed environment. Once deeply anesthetized, animals were decapitated and their brains were quickly removed and transferred to chilled, oxygenated artificial cerebrospinal fluid (ACSF) in which NaCl had been replaced by equivalent osmolarity of sucrose and containing (in mM) sucrose 252 (NaCl 126 in standard ACSF); KCl, 3; $NaH_2PO_4$, 1.25; $MgSO_4$ 7 $H_2O$, 1.3; $CaCl_2$, 2.5; $NaHCO_3$, 26; and glucose, 10, and saturated with a gas mixture of 95% $O_2$ and 5% $CO_2$. Four hundred μm thick slices comprising the nAcb were cut in the parasagittal plane using a vibrating microtome (Campden Instruments). Slices were transferred to a submerged type of recording chamber and continuously superfused with standard ACSF at room temperature (20-22°C) at a rate of 1.5ml/min. The nAcb was visualized under a stereomicroscope (Leica Inc.) using the anterior commissure, the neostriatum, the septum and the ventricles as landmarks based on Paxinos and Watson (1986). The slices were incubated at least one hour before recording.

***Recording*** Whole-cell recording was achieved using the 'blind' patch-clamp technique (Blanton et al., 1989). Pipettes were pulled from thin wall borosilicate capillary glass on a P-87 micropipette puller (Sutter Instrument). The pipettes had a resistance of 3-5MΩ when filled with a solution containing (in mM) potassium gluconate, 140; $MgCl_2$, 2;

$CaCl_2$, 0.1; EGTA, 1.1; HEPES, 10; $K_2$-adenosine trisphosphate (ATP), 2; guanosine trisphosphate (GTP), 0.5 and 0.3% neurobiotin. The pH was adjusted to 7.3 with KOH solution, and final osmolarity was 290-300 mosmol/kg. QX314 (5mM; Alomone Laboratories) was routinely added to the recording pipette solution to prevent voltage-sensitive $Na^+$ channels from generating action potentials.

Whole-cell recordings were made with an Axoclamp 2B amplifier (Axon Instruments) in continuous single-electrode voltage-clamp mode. The output of the amplifier was fed into a LPF 200A DC amplifier/filter (Warner Instruments Corp.) and digitized at 0.5 to 10 kHz with a real-time acquisition system Digidata 1200 (Axon Instruments). Data acquisition was achieved using the pClamp 6.0 software (Axon Instruments). Pipettes capacitance was optimally adjusted before whole-cell configuration was achieved. The resting membrane potential was measured just after rupturing the cell membrane and the offset potential, measured upon withdrawal of the electrode from the cell, was accounted for assuming that it drifted in a linear fashion with time from the start of the recording session. We did not correct for liquid junction potential, which for a pipette containing 140 mM potassium gluconate amounts for an additional potential shift of around –10mV (Spigelman et al., 1992).

***Synaptic stimulation and drugs application*** Excitatory postsynaptic currents (EPSCs) were evoked by 0.1ms, 3 to 6V cathodal pulses delivered at 15 sec intervals to the cortical tissue towards the rostral pole of the nAcb, using a monopolar tungsten stimulating microelectrode placed on the slice superficial layer, 0.5-1.0 mm from the recording electrode. In some experiments, the paired-pulse stimulation with the same

parameters and separated by 50 msec were used to distinguish between pre- and postsynaptic mechanisms. In order to isolate glutamate receptor-mediated EPSCs, all experiments were performed in the presence of (-) bicuculline methiodide (BMI, 10 μM) in bath solution to block $GABA_A$ receptor-mediated synaptic currents. BMI was applied 30 min before obtaining whole-cell configuration to insure a complete diffusion in the slice tissue. In all experiments the EPSCs were recorded from online voltage-clamped potentials between -100 to +40mV in 20mV increment from a holding membrane potential of -70mV. Local application of glutamate (10 mM) onto nAcb was conducted using a patch pipette connected to a Picospritzer (General Valve Corporation) under differential interference contrast and infrared optics using pressure pulses of 15psi lasting 5-10 msec.

The following pharmacological agents were applied through the superfusing ACSF: 6 cyano-7-nitroquinoxaline-2,3-dione (CNQX); (+)-2-amino-5-phosphonopentanoic acid (APV) and (-) bicuculline methiodide all obtained from Tocris (Bristol, UK); dopamine HCl, S-(-)-5-amino-sulfonyl-N-[(1-ethyl-2-pyrrolidinyl)-methyl]-2-methoxybenzamide (sulpiride), (4aR-trans)-4,4a,5,6,7,8,8a,9-octahydro-5-propyl-1H-pyrazolo[3,4-g]quinoline [(-)-quinpirole hydrochloride]; (±)-1-phenyl-2,3,4,5-tetrahydro-(1H)-3-benzazepine-7,8-diol (SKF-38393); R(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1h-3-benzazepine hydrochloride [(+)-SCH-23390]; 9-(tetrahydro-2furyl) adenine (SQ 22536) which have been obtained from RBI (Natick, MA); clozapine from Sigma; Ro-32-0432; H89 and forskolin from Calbiochem (La Jolla, CA). Most drugs were made up as 10 mM stock solutions in distilled water (dopamine

on the day of use) and diluted with ACSF solution to final concentration just before addition to the perfusion medium. The same procedure was used for CNQX except that it was initially dissolved in dimethysulfoxide (DMSO, final concentration 0.1%). Antagonists were applied for at least 15 min before application of agonists. In the cases, of H89 and Ro-32-0432, slices were incubated for 2-4 hours with the antagonists prior to experimentation to allow enough time for the drug to equilibrate within the slice tissue.

***Current measurements*** Data analysis was done using Signal 2.1 software (Cambridge Electronic Design). The amplitude of the evoked synaptic current was plotted as a function of voltage at two points: one at the peak of inward current recorded at -100mV, and another later one at a point when the fast inward current at –100 mV had just decay to base line. This point was usually close to the maximal amplitude of the late component as recorded in the presence of the AMPA/KA receptor antagonist CNQX and no postsynaptic current was observed at this point at holding membrane potential -100 mV under these conditions (Mayer et al., 1984; Mayer and Westbrook, 1987).

***Statistics*** Statistical analysis was performed using Sigmastat 2.0 software (SPSS) and the effects of dopaminergic compounds on evoked EPSCs were tested using paired *t*-test unless otherwise stated (P values of less than 0.05 were considered as statistically significant). All numerical data are expressed as mean ± standard error of the mean (S.E.M). Neurons that could not be unambiguously classified as MS cells using physiological and morphological parameters were excluded from statistical analysis.

## RESULTS

Whole-cell voltage-clamp recording was obtained from 154 physiologically identified MS neurons (O'Donnell and Grace, 1993; Belleau and Warren, 2000; Zhang and Warren, 2002) in slices from rat pups between P1 and P20. The membrane and firing characteristics of MS neurons were similar to those previously reported for animals of comparable age (Belleau and Warren, 2000). In addition, 58 neurons filled with neurobiotin were examined under light microscopy and displayed features that have been previously attributed to MS neurons from animals of similar age (Tepper et al., 1998). All labelled neurons appeared to be located in the core region of the nAcb.

### *Characteristics of glutamatergic EPSCs*

As previously described (Zhang and Warren, 2002), local electrical stimulation in the presence of the $GABA_A$ receptor antagonist BMI evoked an EPSC in all recorded neurons. Typically, the EPSC consisted of a compound glutamatergic EPSC comprising an early and a late component mediated respectively by the activation of AMPA/KA and NMDA receptors.

Figure 1 shows a representative example of an EPSC recorded in a preparation from a P20 animal on which specific glutamatergic antagonists were tested. During the control period (Fig. 1A panel 1), the early EPSC peaked 9 msec after the stimulus onset at a holding membrane potential of –100 mV and the response decayed to baseline within 45 msec. The current-voltage relationship ($I_R$-$V_m$) of the early EPSC was close to linear and reversed at a membrane potential around 0mV (Fig. 1B panel 1). Bath application of the AMPA/KA receptor antagonist CNQX completely abolished the early component of

the EPSC and there was virtually no residual postsynaptic current at all membrane potentials at the latency in which the early response was measured (Fig. 1A panel 2 and Fig. 1B panel 1).

The late component, measured after the early component had decayed, increased at membrane potentials between –100 and –40 mV and reached its maximum usually at –40 mV or –20 mV. At more depolarized membrane potentials, it decreased and reversed polarity around 0 mV (Fig. 1A and Fig. 1B panel 2), a current-voltage relationship typical of NMDA receptor-mediated current. The addition of the NMDA receptor antagonist APV to the superfusing medium completely abolished the late EPSCs (Fig. 1 panel 3), demonstrating that it was mediated by NMDA-type receptors. In the presence of CNQX alone, the NMDAR-EPSC was recorded in isolation showing that measurements of the late component of the EPSC made on the compound EPSC were close to the peak of the NMDAR-EPSC and represented mostly NMDA receptor-mediated current (Fig. 1A panel 2). Also, note there was no residual postsynaptic current in the presence of CNQX and APV, showing that glutamatergic EPSCs were effectively isolated by the addition of BMI to the superfusing medium (Fig 1A panel 4 and Fig. 1B). CNQX and APV were tested together in 4 other neurons producing similar results. In addition, CNQX and APV were tested individually in 17 and 14 neurons respectively producing an inhibition of the early and late components of the response by 91± 2% and 85 ± 5%.

**[Figure 1]**

In most neurons, the effects of dopaminergic agonists and antagonists were assessed at holding membrane potentials usually between -100 and +40mV in steps

Figure 1.

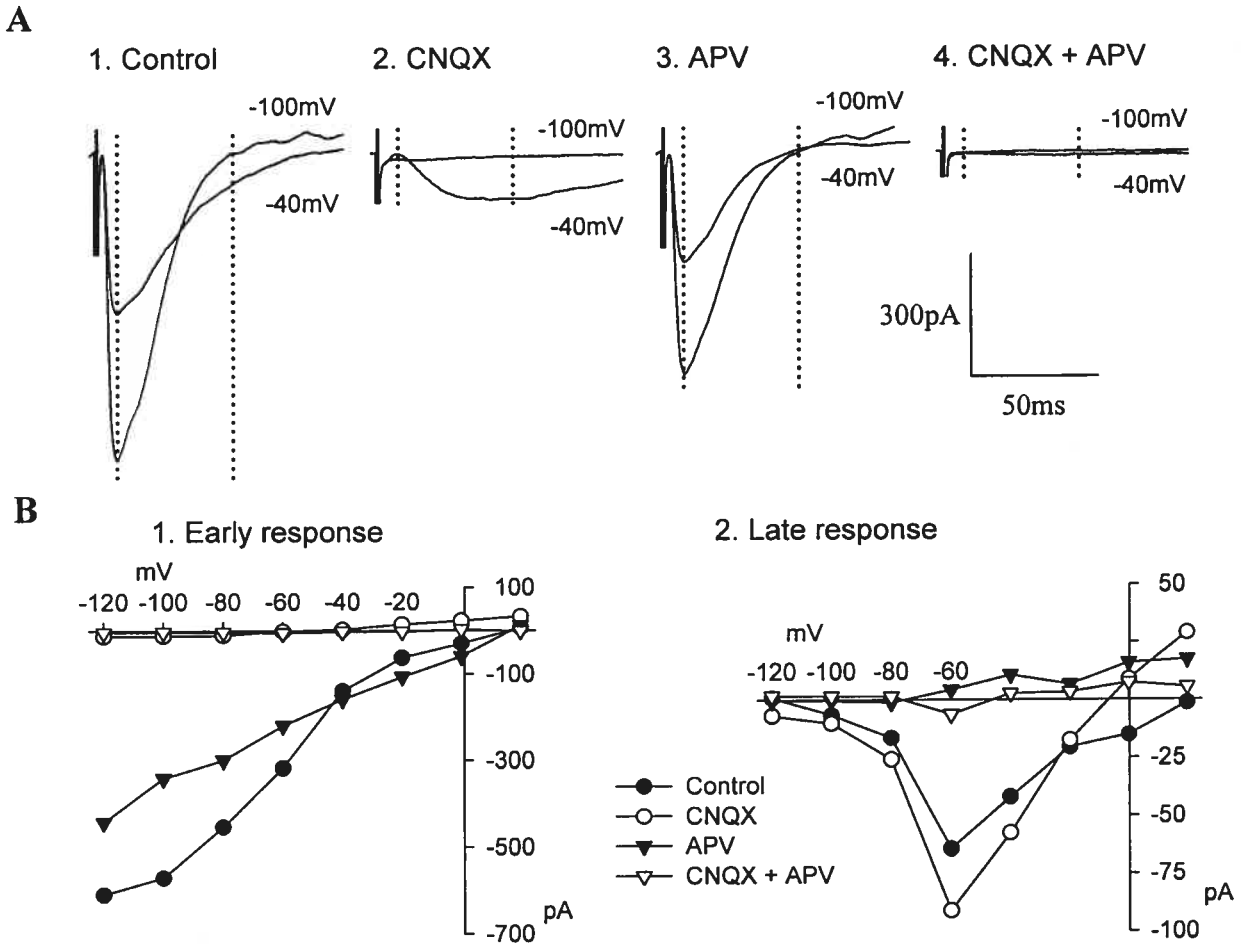


**Figure 1.** Pharmacologically isolated excitatory postsynaptic currents (EPSCs). **A.** Two distinct components of the EPSCs were evoked by local electrical stimulation in the presence of 10 μM of the $GABA_A$ergic antagonist BMI in MS neurons depending on the holding membrane potentials. Before an application of glutamatergic receptor antagonists (**1**); during the presence of CNQX (20 μM) (**2**); during the application of APV (50 μM) (**3**) during the presence of CNQX and APV (**4**). Recordings were obtained in a P20 animal. Current traces represent the average of 6 sweeps. **B.** The $I_R$-$V_m$ relationship of the response between −100 and +40 mV. The early component was measured 9ms after the stimulus as indicated by the left dotted line in **A**. The late component was measured 50 ms after stimulus as indicated by right dotted line in **A.**

of 20mV. The AMPA/KAR-EPSC was measured at the peak of the early component of the EPSC at a holding membrane potential of –100 mV, when the amplitude of the late component was minimal (left vertical dotted lines in Fig. 1A) whereas the effects on NMDA receptor-mediated currents were measured at a latency at which the early component recorded at a holding membrane potential of –100 mV had decayed (right vertical dotted lines in Fig. 1A).

***Effects of dopaminergic agonists***

Exposure of slices to 50 μM of dopamine for 8-10 min resulted in a perceptible decrease in the amplitude of both CNQX- and APV-sensitive current components of evoked EPSCs. This decrease in EPSC amplitude significantly occurred in holding membrane potentials between –100mV and –20mV and was completely reversible and repeatable once dopamine was washout and reapplied. A representative example of this effect is shown in Figure 2A. In this case, the early and late components of the EPSCs recorded at –100mV and –20mV, respectively, were reversibly reduced by 41% and 72% during the application of DA. Similar results were obtained in 78 other neurons, and DA produced no effects on the EPSC in just one case. The effects of DA on the early and late components of the EPSC as a function of holding membrane potential are summarized in Figure 2B. The amplitude of the early component of the EPSC was significantly reduced at holding membrane potentials between –100mV and –20mV by an average of 51-45% as well as at positive membrane potentials. DA also produced a reduction of the late component of the EPSC, which were generally of larger magnitude than that observed on the early component, averaging 66-57% between –40mV and 0 mV. **[Figure 2]**

Figure 2.

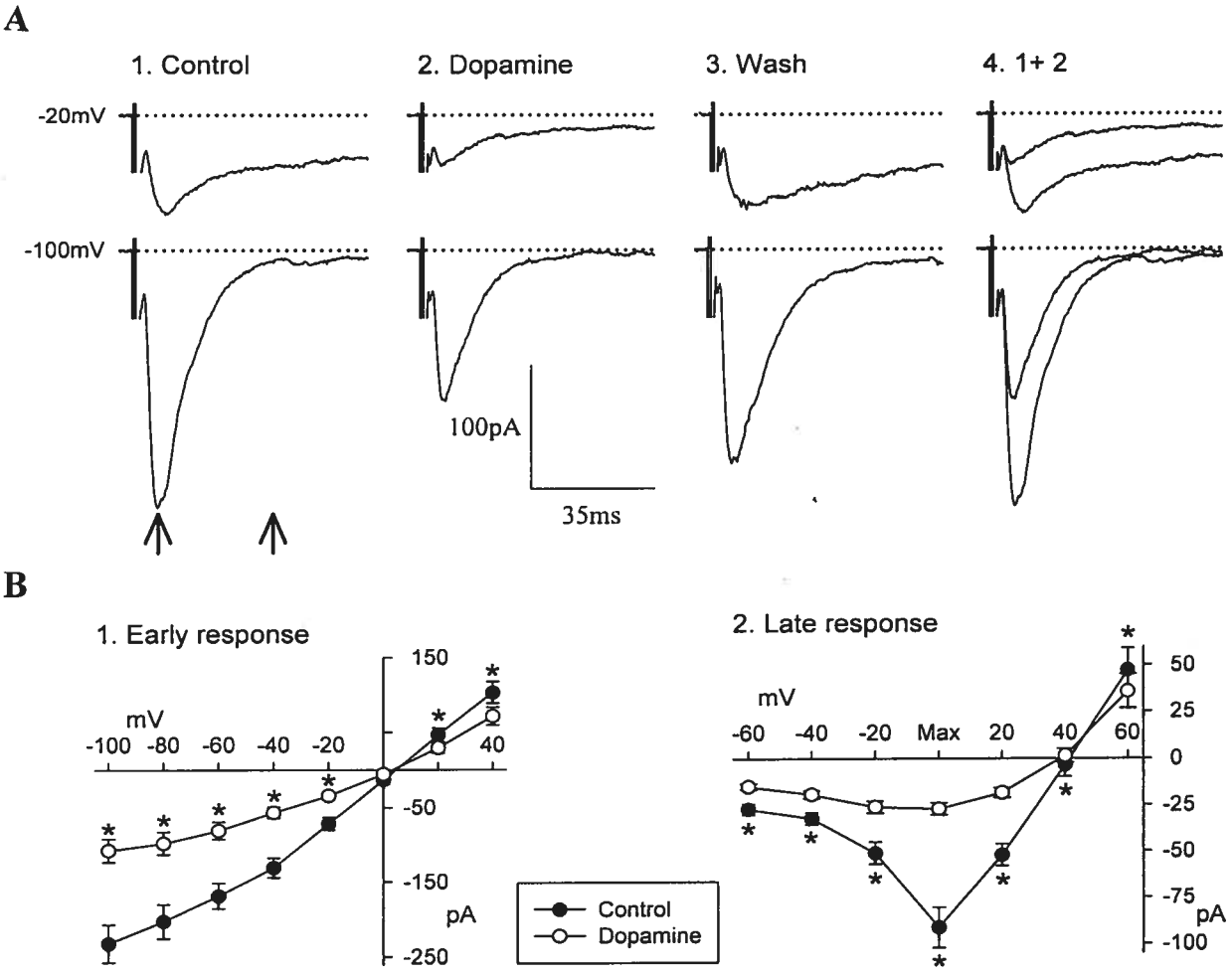


**Figure 2.** Effect of dopaminergic agonist on the EPSCs. **A.** Current traces of the response evoked by single electrical stimulus recorded at holding membrane potentials of –100mV and -20mV. Before (**1**), during (**2**), and after (**3**) superfusion with dopamine (DA 50 μM). The overlay (**4**) shows the responses before and during DA application. Current traces represent the average of 6 sweeps. Recordings were obtained in a slice from a P7 animal. The arrows in (**1**) indicate where the early and late responses were measured. **B.** Average $I_R$-$V_m$ relationship of the early (n= 80; **1**) and late (n=80; **2**) recorded before and during superfusion with DA. Asterisks indicate a statistically significant difference between control and agonist treatment at this holding membrane potential (Student's *t*-test, **P*<0.05).

***Dopamine reduced the ratio of NMDAR-EPSCs to AMPA/KAR-EPSCs***

In the study on compound EPSCs, we found that both early and late components of EPSCs were significantly inhibited by 50 μM of DA. To investigate the efficacy of DA on AMPA/KAR-EPSCs and NMDAR-EPSCs, we also examined the ratio of the late component to the early component of the EPSCs.

In 67 neurons, we found that application of 50 μM DA significantly reduced the late to early component of EPSC ratio by an average of 50% from 28 ± 2% during control to 14 ± 3% with DA (paired *t*-test, $P< 0.01$, n = 67, Fig. 3B). This effect was present throughout the developmental period studied (Fig. 3). Our data demonstrates that DA decreased the late to early component of EPSCs ratio in all of ages and that DA more effectively reduces NMDAR-EPSCs than AMPA/KAR-EPSCs rather than decreasing them both equally.

**[Figure 3]**

To further test the inhibitory effect of DA on NMDAR-EPSCs, we compared its inhibitory effect with a specific NMDA receptor antagonist, APV on the late component of the EPSCs in 4 cells. We found that DA was as potent as APV in reducing the late component of the EPSCs (144 ± 54 pA in control, 26 ± 13 pA in DA, average inhibition 82%: 117 ± 43 pA in control and 15 ± 8 pA in APV, average inhibition 87% (Fig. 4C). Figure 4A show a representative example of the effects produced by DA and APV in the same late component of the EPSCs.

**[Figure 4]**

Figure 3.

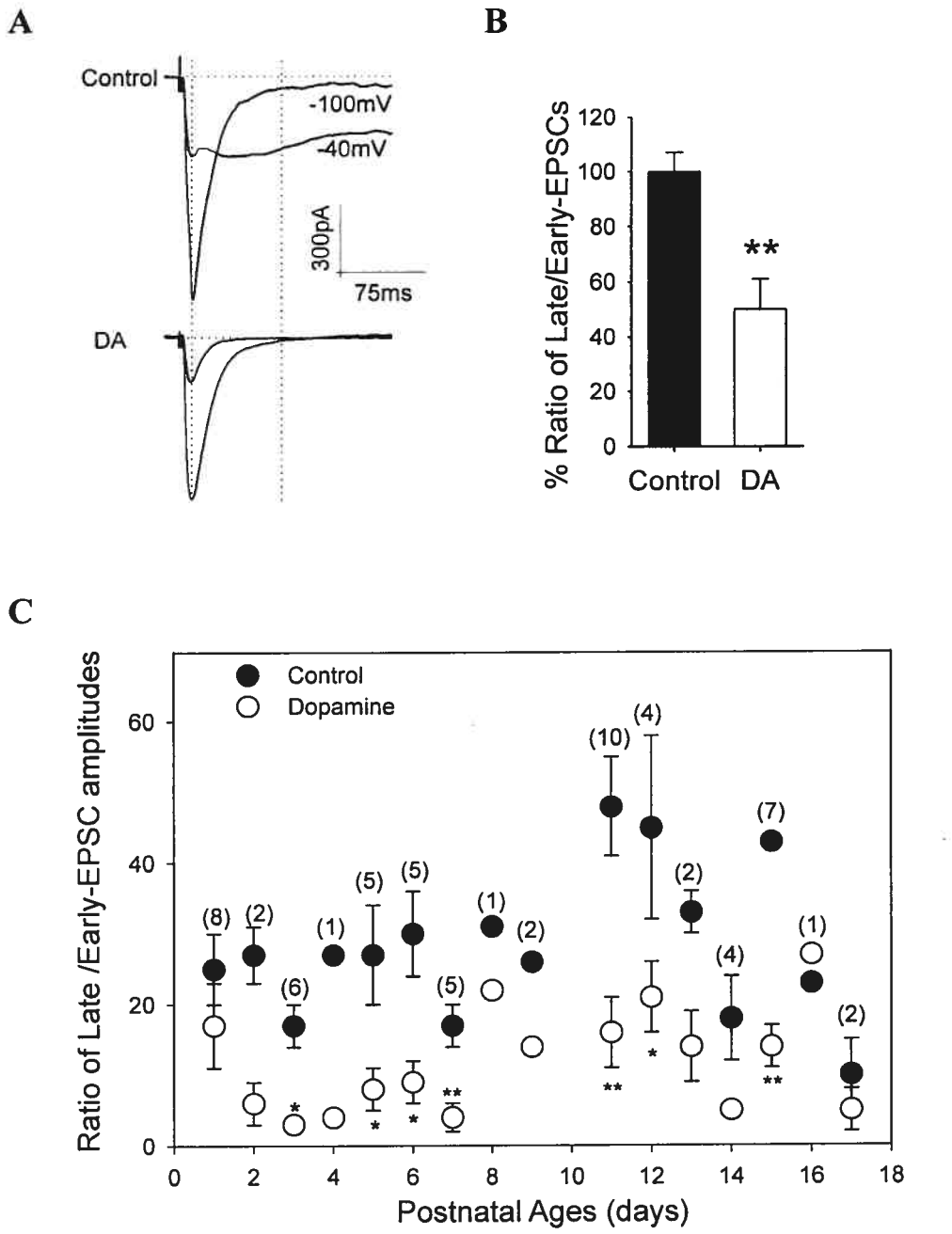


**Figure 3**. DA (50 μM) decreased ratio of late to early component of the EPSCs. **A.** Representative traces taken from holding membrane potentials of –100mV and -40mV in a MS neuron from a P15 animal slice in the presence or absence of DA (50 μM) respectively. **B.** Averaged late to early component ratio from 67 neurons during control and DA application (Student's *t*-test; $P<0.01$, n =67). **C.** Late to early component ratio as a function of postnatal age (Student's *t*-test; n = 65; ** p< or = 0.01; * p< or = 0.05). The number of cell at each age is indicated in the brackets. DA is specifically preferential to attenuate NMDAR-EPSCs. Dotted lines in **A** indicate the sites where EPSCs were measured.

Figure 4

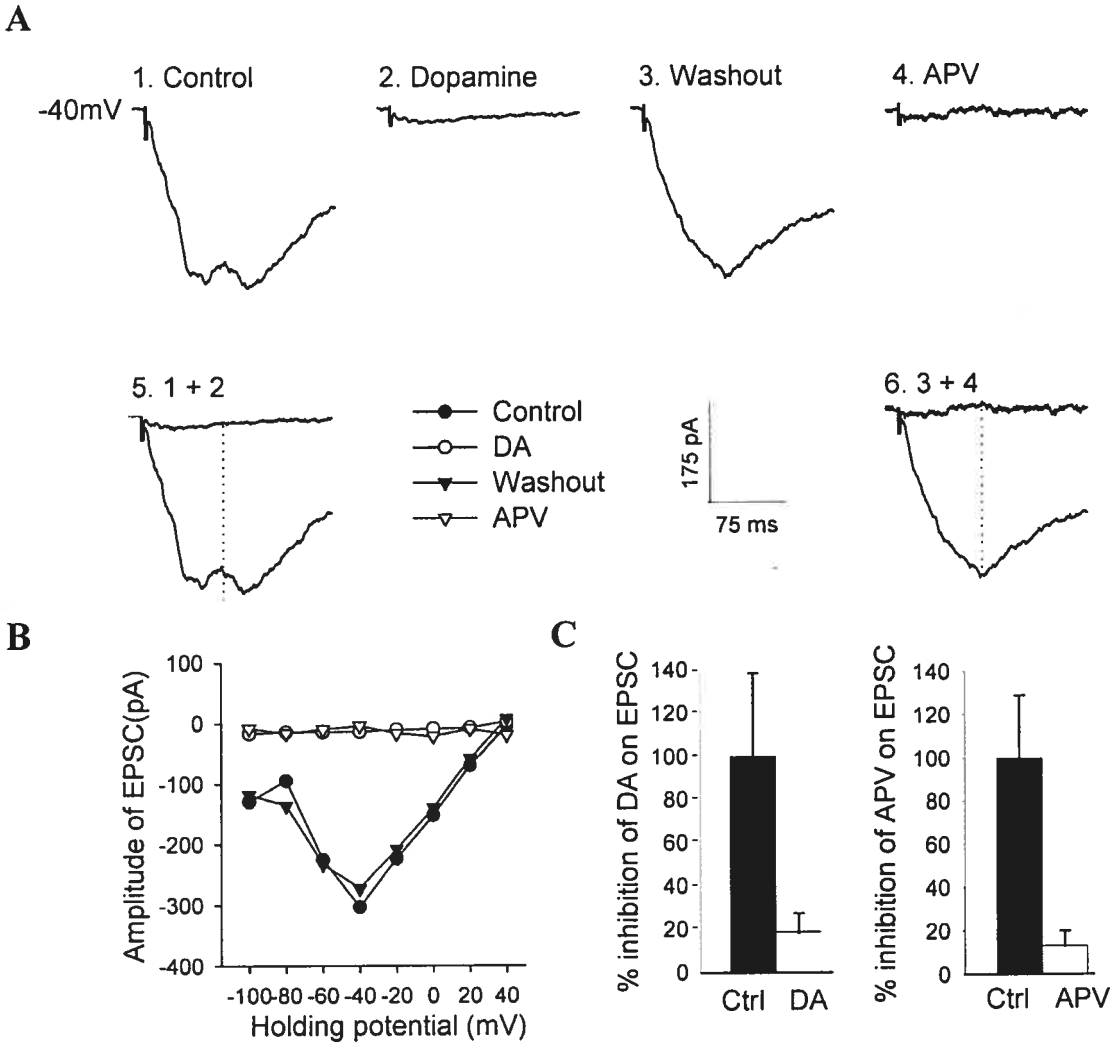


**Figure 4.** The inhibitory effects of DA and APV on late component of compound EPSCs. **A.** Inhibitory efficacy of DA (50 μM) and APV (50 μM) is shown in a representative neuron in which evoked EPSC was mainly mediated by NMDA receptors. Before (**1**), during (**2**), and after (**3**) DA perfusion, **4** during APV; overlay (**1+2, 5**) and overlay (**3+4, 6**). **B.** The $I_R$-$V_m$ relationship for the representative neuron. Dotted lines in **A** indicate sites where current amplitudes were measured and displayed in **B**. **C.** % inhibitions produced by DA and APV on late EPSCs were compared in 4 neurons at a holding membrane potential of –40 mV between the effect of DA and APV (Student's t-test, $P > 0.05$, n= 4).

***DA inhibited both isolated NMDAR-EPSCs and AMPA/KAR-EPSCs***

Because it was reported that DA exerted opposite rather than a parallel modulatory effects on AMPA/KAR-EPSCs and NMDAR-EPSCs i.e. DA potentiated NMDAR-EPSCs and depressed AMPA/KAR-EPSCs in the nAcb (Harvey and Lacey, 1997; Chergui. and Lacey, 1999) whereas others reported that DA produced no effect on NMDAR-EPSCs (Nicola and Malenka, 1998; Beurrier and Malenka, 2002). We tested the effect of DA on pharmacologically isolated AMPA/KAR-EPSCs and NMDAR-EPSCs using CNQX (20 μM) and APV (50 μM) in the presence of the $GABA_A$ receptor antagonist BMI (10 μM). The studies on isolated EPSCs further confirmed that DA (50 μM) had a profound inhibitory effect on NMDAR-EPSCs. In the presence of AMPA/KA receptor antagonist, CNQX, DA potently inhibited NMDAR-EPSCs in all 5 isolated neurons. In control recordings, the NMDAR-EPSCs amplitude averaged –77 ± 8 pA. During bath application of 50 μM DA for 8 min, the amplitude of the NMDAR-EPSCs was significantly reduced to 7 ± 5 pA (the peak amplitude of NMDAR-EPSC was decreased by 91%, paired *t*-test. $P< 0.01$, n = 5, Fig. 5C). Figure 5A shows a representative cell in which the isolated NMDAR-EPSC was strikingly inhibited.

**[Figure 5]**

DA (50 μM) also significantly reduced the amplitude of stimulus-evoked AMPA/KAR-EPSCs, but only by 40% of control. In control recordings (holding membrane potential –100mV), the EPSC amplitude averaged -241± 45 pA. During bath application of 50 μM DA for 8 min, the amplitude of AMPA/KAR-EPSCs was

Figure 5.

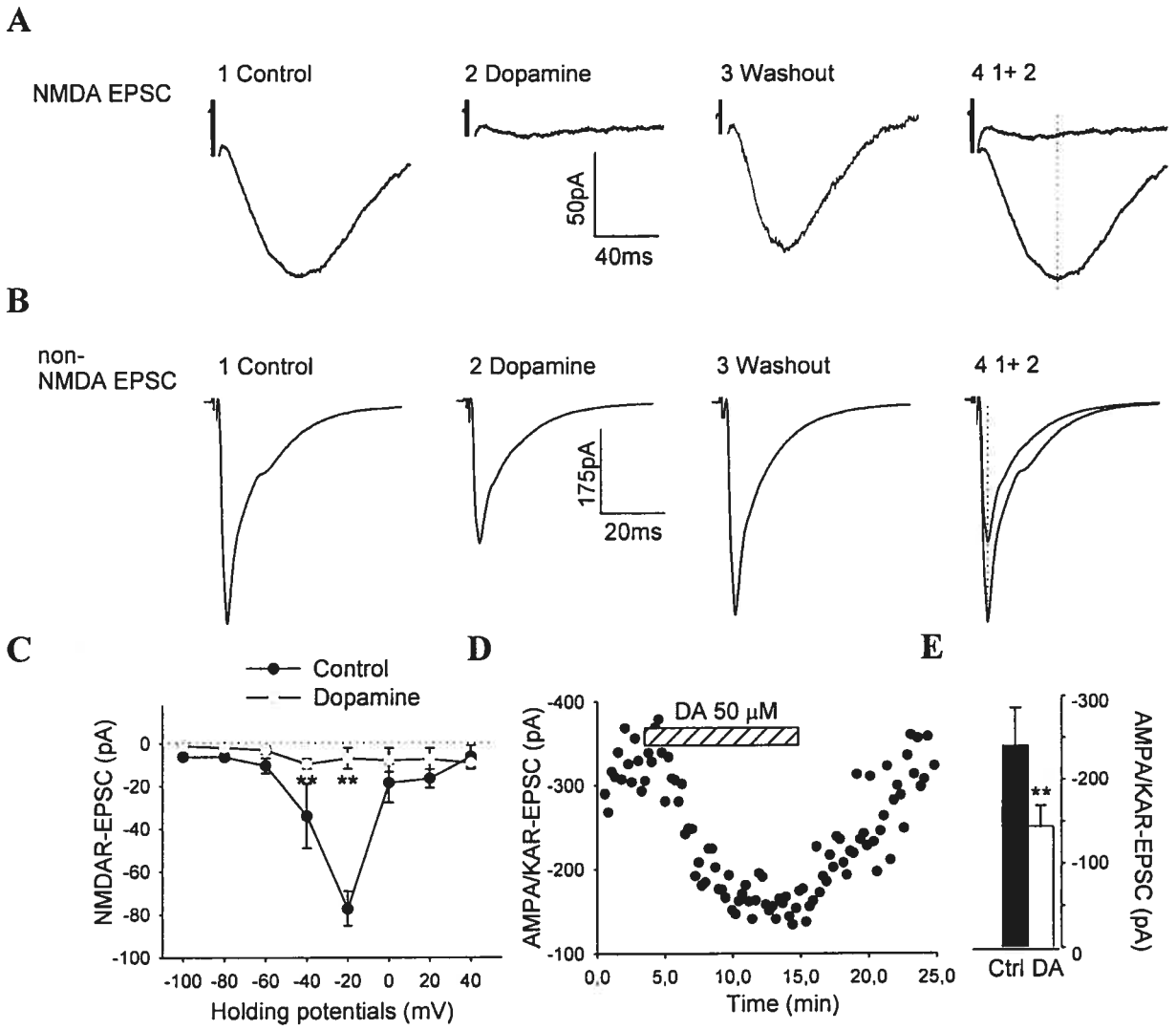


**Figure 5.** DA inhibited both NMDAR-EPSCs and AMPA/KAR-EPSCs. **A.** DA (50 μM) potently inhibited NMDAR-EPSCs in the presence of CNQX. Original traces evoked by electrical stimulus were taken from holding membrane potential –20 mV and represent the average of 6 sweeps. BMI (10 μM) and CNQX (20 μM) were present in the superfusing medium throughout recording. Before (**1**), during (**2**), and after (**3**) DA. **4** is an overlay of **1** and **2**. **B.** DA (50 μM) also significantly inhibited AMPA/KAR-EPSCs isolated with APV (50 μM) and BMI (10 μM). Before (**1**), during (**2**) after (**3**) DA (50 μM), and **4** is an overlay from **1** and **2**. **C.** The $I_R$-$V_m$ relationship at membrane potential from –100mV to 40mV was measured in the absence and presence of DA on isolated NMDA currents. DA (50 μM) used in bath for 10 min attenuated most of NMDAR-EPSCs at all holding membrane potentials (Student's *t*-test, n = 5, ** $P<0.01$). The dotted lines in panels **A** and **B** label the sites for the measurement of $I_R$-$V_m$ relationship. **D.** Time course of DA inhibition of AMPA/KAR-EPSCs from panel **B**. **E.** Summary of the effect of DA (50 μM) on AMPA/KAR-EPSCs at holding membrane potential –100 mV (Student's *t*-test, n = 7, ** indicates $p< 0.01$).

significantly reduced to -144 ± 25pA (Fig. 5E, paired *t*-test, $P<0.05$; n = 7). Figure 5B shows a typical example of the inhibitory effect of DA on AMPA/KAR-EPSCs at a holding membrane potential of –100mV traces and Figure 5D shows time course of the effect.

***Inhibition of DA on EPSCs is mediated by a $D_1$-like receptor***

To identify the DA receptor subtype responsible for the inhibition of EPSCs, we examined the effects of $D_1$- and $D_2$-like receptor agonists and antagonist on stimulus-evoked EPSCs. Like DA, SKF 38393 (10 μM), an agonist of $D_1$-like DA receptors, decreased the early EPSC peak amplitude by 43% of control at holding membrane potential –100mV (-304 ± 131 pA in control and –173 ± 76 pA, in SKF 38393, n = 5) and the late EPSC amplitude by 53% at holding membrane potential –40mV (-51± 23 pA in control and 24 ±15 pA in SKF 38393, see table I). Figure 6A shows a representative example of the inhibitory effect of SKF 38393 on the early and late components of the EPSCs. In contrast, the $D_2$-like receptor agonist quinpirole failed to suppress EPSCs in the same concentration (n=4; data not shown). Concomitantly, the $D_2$-like receptor antagonist sulpiride (10 μM) failed to prevent the 40% depression-induced by DA (n = 4), which is not different from the observed decline in the absence of the antagonist (-224 ± 22 pA in control, and –112 ± 14 pA in DA at –100 mV, n = 4). In addition, clozapine (10 μM), an antagonist of $D_2$-like dopamine receptors and certain 5-HT receptors, failed to block the inhibitory effect of DA (Student's *t*-test, $P > 0.05$, n = 9, see table I), further excluding the possibility that $D_2$-like receptor may be involved. However, the $D_1$-like receptor antagonist SCH 23390 (10 μM) antagonized the depressant action of DA (50

μM) to 87% of control (-205 ± 44 pA in control and –179 ± 33 pA in DA in the presence of SCH 23390) at holding membrane potential –100 mV, and to 91% of control (-65 ± 15 pA in control and –59 ± 15 pA in DA) at –20 mV (Student's t-test, $P > 0.05$, n = 9), respectively. Figure 6C shows a representative cell in which $D_1$ receptor antagonist SCH 23390 (10 μM) blocked the inhibitory effect of DA on compound EPSCs, whereas DA alone suppressed much of the EPSC. Figure 6D summarizes the effect of DA in the presence or absence of $D_1$ receptor antagonist SCH 23390 at holding membrane potential –100 mV and –20 mV in 9 neurons. We also found that low concentrations of SCH 23390 (1 or 5 μM) only decreased partially the inhibitory effect of DA (n = 20, data not shown).

**[Figure 6]**

We attempted to characterize the signal transduction pathways mediating the effects of DA (Snyder et al., 1998). H89 (20 μM), a cell-permeable, selective and potent inhibitor of protein kinase A (PKA) in both pipette and bath failed to block the inhibitory effect of DA on both early and late components of the EPSCs in the presence of H89 (n = 7, see table I). Ro-32-0432 (10 μM), a selective cell-permeable inhibitor of protein kinase C (PKC) could not abolish the inhibitory effect of DA (n = 3, see table I). We also examined the role of the cAMP pathway by activating adenylate cyclase (AC) with forskolin. Forskolin (10 μM) increased AMPA/KAR-EPSCs and NMDAR-EPSCs, and also appeared to partially antagonize the inhibitory effect of DA (n = 5, see table I), but, QS22536 (100 μM), an antagonist of AC, had no effect on the inhibitory effect of DA (n = 3, see table I). We also tested the effect of DPCPX (500 nM), an $A_1$ receptor

Figure 6.

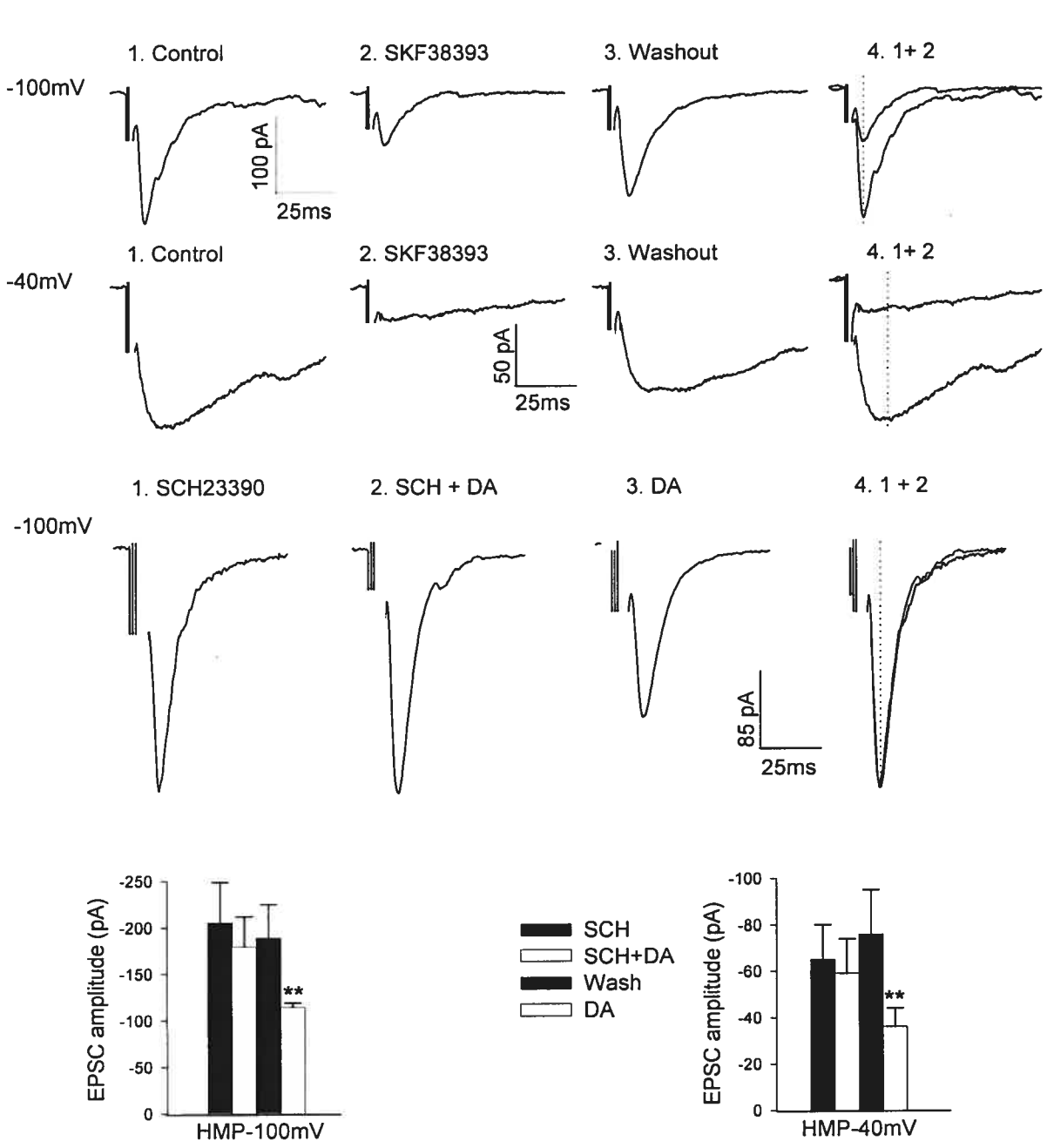


**Figure 6.** The inhibitory effect of DA on the EPSCs was mediated by $D_1$-like receptor. **A.** Representative cell in which $D_1$-like receptor agonist SKF 38393 (10 μM) mimicked the inhibitory effect of DA on both the early (-100mV) and late (-40mV) components of the EPSCs. Before (**1**), during (**2**), after (**3**) SKF 38393. **4** is an overlay of **1** and **2**. **B.** The effect of DA was blocked by $D_1$-like receptor antagonist. In the presence of $D_1$-like receptor antagonist, SCH23390 (10 μM, **1**); adding DA (50 μM for 10 min in the presence of SCH23390, **2**); reapplying DA after washout of SCH23390 and DA, **3**); **4** is an overlay of **1+2** where the inhibitory effect of DA was blocked by SCH23390. **C.** Summary of the inhibitory effect of DA in the presence or absence of SCH23390 at holding membrane potential –100 and –40mV for 9 cells. The dotted lines in **A** and **B** indicates the sites where the amplitudes of the EPSCs were measured (Student' *t*-test, **P< 0.01).

Table I

The effects of DA on EPSCs in the presence of different antagonists and forskolin

| | | | Early EPSCs (pA) (-100mV) | | Late EPSCs (pA) (-20mV) | |
|---|---|---|---|---|---|---|
| Antagonists | | n | Control | DA(50μM) | Control | DA(50μM) |
| Sulpiride | 10μM | 4 | -150 ± 26 | -92 ± 30 | -59 ± 6 | -37 ± 6 |
| Clozapine | 10μM | 9 | -228 ± 34 | -91 ± 20 | -87 ± 24 | -21 ± 14 |
| QS22536 | 100μM | 3 | -165 ± 8 | -14 ± 7 | -50 ± 9 | -8 ± 0 |
| H89 | 20μM | 7 | -331 ± 124 | -152 ± 67 | -141±58 | -39 ± 25 |
| Ro320432 | 10μM | 3 | -158 ± 29 | -92 ±20 | -51 ± 20 | -13 ± 4 |
| Forskolin | 10μM | 4 | -266 ± 38 | -220 ± 12* | -153 ± 21 | -102 ± 12* |

* indicates P>0.05 (student' *t*-test)

antagonist (see Harvey and Lacey, 1997) in a few cells and no significant effect was found (data not shown).

***Pre and postsynaptic mechanisms are involved in the attenuation of EPSCs by DA***

The mechanism underlying DA inhibitory effect on EPSCs may involve a decrease in glutamate release (presynaptic) and/or change in membrane conductance (postsynaptic). To identify the site of action of DA, we compared different characteristics of the EPSCs before and during DA administration.

An alteration in the paired-pulse ratio (PPR) is thought to imply presynaptic mechanism of action (Zucker, 1989; Regehr and Stevens, 2001). To determine if the locus of dopamine-dependent inhibition of the EPSC amplitude in MS neurons is pre- or postsynaptic, we examined the effects of DA on the paired-pulse stimulation-evoked EPSCs (stimuli with 50 ms interpulse interval) comparing the change of PPR i.e. the $2^{nd}$ response to the $1^{st}$ response (EPSC2/EPSC1) at a holding membrane potential –100mV. We found that in the presence of DA both the first and second evoked EPSCs were decreased but a relatively greater reduction in the amplitude of the first EPSC was observed resulting in an increase in the PPR (in control recordings, PPR was 1.3 ± 0.05; during bath application of DA, it was 1.6 ± 0.14, paired *t*-test, $P< 0.05$, n = 12, Fig. 7A and A'). However, the PPR of the late component recorded at a holding membrane potential of –40mV was not altered during application of DA. The PPR was 2.2 ± 0.14 in control recordings condition, while during bath application of DA, PPR was 2.3 ± 0.14 (Fig. 7B and B', paired *t*-test, $P> 0.05$, n = 12). EPSCs induced at a holding membrane potential of –40mV are largely mediated by NMDA receptors. The fact that the PPR of

the late response does not change suggests that the inhibition of NMDAR-EPSCs by DA is mainly mediated by postsynaptic mechanisms. This implies that the major locus of inhibition by DA may be different for AMPA/KAR and NMDAR-EPSCs.

Change in membrane current conductance is considered to be one of criteria for a postsynaptic effect. We measured the membrane conductance before and during the application of DA (50 μM) at eight holding membrane potentials in 50 neurons and found no statistically significant change in membrane conductance during the application of DA at any membrane potential tested (Fig. 7C, in control recordings, membrane conductance was 33 ± 4 pA, during bath application of DA, membrane conductance was 34 ± 4 pA at holding membrane potential –100mV; in holding membrane potential –40mV, control membrane conductance was 16 ± 2 pA, while bath application of DA, membrane conductance was 23 ± 4 pA. paired *t*-test, $P > 0.05$, n = 50). This suggests that no intrinsic property of the membrane altered during the application of DA.

Change in the time course of synaptic response may be an indicator for detecting a presynaptic or postsynaptic effect. We measured the time course of synaptic responses for AMPA/KAR-EPSCs in 9 cells before and during application of DA. The results indicate that DA did not produce significant changes in the decay time ($\tau$) of the evoked EPSCs (in control: 10.94 ± 1.8 ms, in DA 12.64 ± 3.1 ms at holding membrane potential –100 mV, paired *t*-test, n = 9, $P > 0.05$). The decay of the EPSC in the presence of DA could be perfectly fitted with the controls (data not shown).

Steady-state current-voltage curves generated by changing membrane potentials showed that DA did not change the I-V relationship configuration over the voltage range

tested in early compound EPSCs (from –100 to +40 mV) although EPSCs were depressed to some extent (Fig. 2, n = 80).

Finally, effects of DA (50 μM) on postsynaptic response evoked by pressure application of glutamate (10 mM) were studied at a holding membrane potential of –100mV. DA (50 μM) neither reduce the mean peak amplitude of glutamate-evoked response nor changed the kinetic curve of the currents, indicating the depression of EPSCs produced by DA was not mediated by postsynaptic mechanisms on glutamate receptor (Fig. 7 D and D'. 120 ± 10 pA in control; 121± 10 pA in DA, Student's *t*-test, $P>0.05$, n = 5).

In summary, DA decreased the NMDAR-EPSCs to AMPA/KAR-EPSCs ratio in all age groups but did not change the PPR of NMDAR-EPSCs at a holding membrane potential of –40mV, suggesting that the inhibition of NMDAR-EPSCs by DA might be mediated predominantly by postsynaptic mechanisms. DA significantly altered the PPR of the AMPA/KAR-EPSC at a holding membrane potential of –100 mV. In addition, there was no change in membrane conductance at any holding membrane potential levels and no effect of DA was observed on glutamate-induced EPSC at holding membrane potential of –100mV, suggesting that the inhibition of DA on AMPA/KAR-EPSC occurred at presynaptic sites.

**[Figure 7]**

Figure 7.

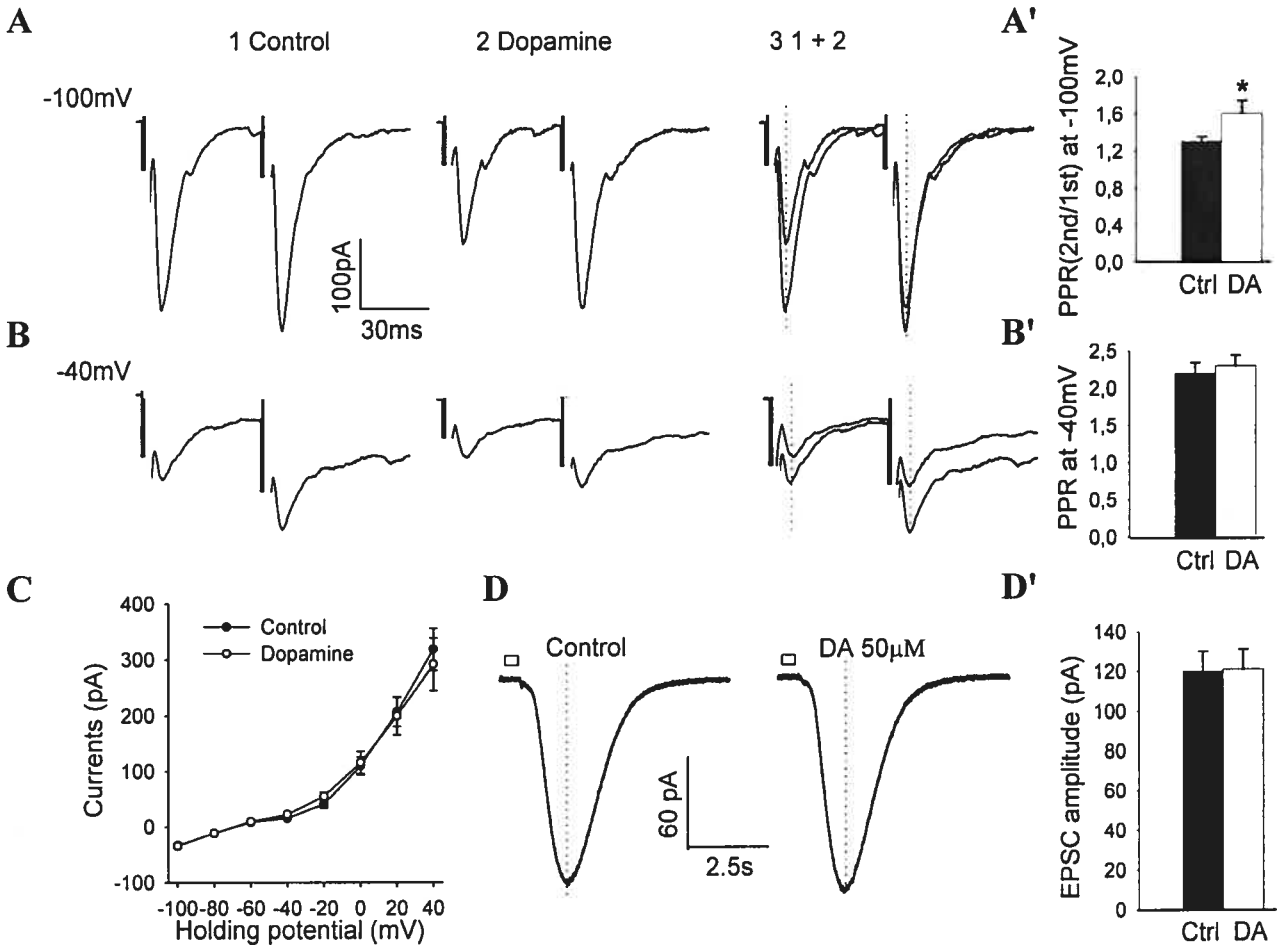


**Figure 7.** DA inhibited paired-pulse-induced EPSCs and increased PPR at holding membrane potential of –100mV, but not at –40mV. **A.** Before (**1**); during (**2**) DA (50 μM). **3** is an overlay of **1** and **2**. **A'**: Summary for 12 cells at holding membrane potential of –100mV. **B.** DA had no significant effect on PPR at holding membrane potential –40mV. **B'**: Summary for 12 cells at –40mV. **C**: The effect of DA (50 μM) on membrane current conductance measured in 50 cells at eight holding membrane potentials between –100mV and +40mV. **D.** The effect of DA (50 μM) on glutamate injection-induced EPSCs in the presence of TTX (1 μM) in perfusing medium. **D'**: Summary of 5 cells for **D** (Student' *t*-test, * P< 0.05). Dotted line indicates the sites where the amplitude of the currents was measured.

## DISCUSSION

Our results support the notion that the EPSC evoked by local stimulus in the nAcb is composed of both early and late components. 50 μM of DA strongly inhibited NMDAR-EPSCs, whereas only slightly but significantly, reduced the amplitude of AMPA/KAR-EPSCs by acting on $D_1$-like receptors. Previous studies have shown that depression of EPSCs or EPSPs produced by DA was mediated by AMPA/KA receptor and our findings are consistent with these results (Higashi et al., 1989; Pennartz, et al., 1992; Harvey and Lacey, 1996, 1997; Nicola and Malenka, 1997, 1998). Our finding that DA produced a much stronger inhibition of NMDAR-EPSCs is, to our knowledge, the first report to demonstrate a preferential depression of NMDAR-EPSCs by DA in the nAcb. In contrast with previous studies, we did not find that the inhibitory effect of DA involved PKA or PKC pathways under our experimental conditions.

### *DA inhibited both NMDAR-EPSCs and AMPA/KAR-EPSCs in the nAcb*

Intra-nAcb infusion of amphetamine reduces levels of extracellular glutamate, suggesting that the dopaminergic system may inhibit glutamate release (Kalivas and Duffy, 1997). Although no axo-axonal synaptic contacts between dopaminergic terminals and limbic cortical afferents exist, there is considerable evidence to indicate that their inputs terminate on the same spines of MS neurons and often in close apposition (Smith and Bolam, 1990; Bouyer et al., 1984; Sesack and Pickel, 1990). This indicates that they interact on a common target-MS neurons to modulate the function of MS projecting neurons and that dendritic spines of MS neurons is a potential site for

physiological interactions between DA and glutamate in the nAcb (Antonopoulos et al., 2002).

DA receptors expression changed during postnatal development in the nAcb (Teicher et al., 1991; Lu et al., 1998; Schwartz et al., 1998; Tarazi et al., 1999; Antonopoulos et al., 2002). The density levels of $D_1$-like receptor increases from P7 to P28, then declines by 20-40% after P35 to remain unchanged until P60. Excessive $D_1$-like receptors are eliminated during maturation of the nAcb (Tarazi et al, 1999). The major finding of the present study is that the activation of DA receptors ($D_1$-like) preferentially inhibited NMDAR-EPSCs in the nAcb *in vitro* during postnatal development. Our conclusion is based on several findings: (1) the ratio of NMDAR-EPSCs to AMPA/KAR-EPSCs amplitude was significantly decreased by DA; (2) the effect of DA on NMDAR-EPSCs was as pronounced as that of the NMDA receptor antagonist, APV, and (3) the inhibitory efficacy of DA on isolated NMDAR-EPSC amplitudes in the presence of CNQX and BMI was over 90% of the control. However, the present finding contradicts the results obtained by Harvey and Lacey (1997) who reported that DA, via the activation of $D_1$-like receptor, enhanced NMDAR-EPSCs in the nAcb slices. Still, others have not observed this effect (Nicola and Malenka, 1998; Beurrier and Malenka, 2002). The conclusion for potentiation of NMDAR-EPSCs by DA (Harvey and Lacey, 1997) was based on the presence of $A_1$ receptor antagonist, DPCPX, but we could not replicate these finding. However, the actual effect of DA on NMDAR-EPSCs was inhibitory in the absence of DPCPX (Harvey and Lacey, 1997). Chergui and Lacey (1999) again reported that $D_1$ agonist increased NMDA inward

current in 50% of the cells and this enhancement was blocked by Ro-32-0432, a PKC inhibitor. This is in contrast with our present results. The discrepancy in the effect of DA on NMDAR-EPSCs in our study and others are perhaps due to the methods used in different studies or to the fact that our study was concluded during early postnatal development. Chergui and Lacey (1999) elicited NMDA currents by injecting NMDA while membrane potentials was held at –90 mV. However, in our experimental protocol we found neither NMDAR-EPSCs at comparable holding membrane potential could be evoked nor the NMDA current was pharmacologically isolated. However, inhibition by DA of both AMPA/KAR- and NMDAR-EPSCs is completely consistent with the findings in the subicular nucleus (Behr et al., 2000) and in striatal MS neurons (Lin et al., 2003). Inhibitory effect of DA on NMDA currents has also been demonstrated in striatal and hippocampal neurons (Lee et al., 2002).

***Both pre- and postsynaptic mechanism were involved in inhibition of EPSCs by DA***

DA receptors are distributed on both the presynaptic glutamatergic terminals and postsynaptic membrane of MS neurons in the nAcb (Lu et al., 1998; Schwartz et al., 1998; Tarazi et al., 1998; Antonopoulos et al., 2002). Therefore, DA may exert its inhibitory action on stimulus-evoked EPSCs by either an alteration of the postsynaptic glutamate receptors or a decrease in presynaptic glutamate release, or a combination of both pre- and postsynaptic mechanisms.

Our findings suggest that the synaptic mechanisms by which DA depresses NMDAR-EPSCs and AMPA/KAR-EPSCs are complex and may be somewhat different. We examined the changes in the PPR during DA application as an indication of pre- or

postsynaptic mechanism. In the paired-pulse paradigm of DA inhibition, if DA acts presynaptically to reduce the probability of glutamate release from presynaptic terminals, then the ratio of the second EPSC to the first EPSC ($EPSC_2/EPSC_1$) amplitude should be altered. In contrast, if DA acts postsynaptically, the amplitude of $EPSC_1$ and $EPSC_2$ should be reduced to the same degree, and therefore the ratio would remain unchanged (Kline et al., 2002). Bath application of DA greatly reduced EPSC, but had less effect on $EPSC_2$ at a holding membrane potential of –100mV, suggesting that DA promotes a decrease in presynaptic quantal release, rather than a decrease in postsynaptic glutamate sensitivity of AMPA/KA receptor. DA significantly altered the paired-pulse depression ratio, but neither changed membrane conductance nor effected the extrinsic glutamate-induced currents at holding membrane potential of –100mV, suggesting that DA inhibits AMPA/KAR-mediated current through presynaptic mechanisms. Our results are consistent with those of previous investigators studying DA inhibition on EPSCs in the nAcb (Nicola and Malenka, 1997, 1998; Harvey and Lacey, 1996, 1997; Pennartz et al., 1992; Higashi et al., 1989). In addition, PPR changes attributed to DA application in relatively hyperpolarized membrane potentials have also been observed in the nucleus of the solitary tract (Kline et al., 2002), in subicular neurons (Behr et al., 2000), in supraoptic nucleus (Price and Pittman, 2001) and in parabrachial nucleus (Chen et al., 1999). Paired-pulse facilitation is due to residual free $Ca^{2+}$ in the presynaptic terminal left unbuffered from the first pulse and summates with $Ca^{2+}$ influx from the second stimulus, enhancing the probability of transmitter release with the second pulse. Therefore, the magnitude of the second response is typically larger (Santschi and Stanton,

2003). A change in the ratio of these amplitudes by DA, in the absence of changes in postsynaptic input resistance, is thought to reflect a presynaptic mechanism (Debanne et al., 1996; Zucker, 1989; Zucker and Regehr, 2002; Kline et al., 2002). However, in testing PPR at a holding potential of –40mV we found that DA neither increased nor decreased PPR at –40mV, i.e. the amplitude of $EPSC_1$ and $EPSC_2$ were reduced to the same degree. No significant PPR alteration at this holding membrane potential suggests that the inhibition of NMDAR-EPSCs by DA may not be mediated by a presynaptic mechanism and could also involve postsynaptic mechanisms.

The larger effect of DA on NMDAR-EPSC may be due to either a specific postsynaptic inhibitory action of DA on NMDA receptors or may be related to a higher affinity of glutamate for AMPA/KA receptors than for NMDA receptor. In the latter case, a low concentration of glutamate would activate more AMPA than NMDA receptors so that the ratio of NMDAR-EPSCs to AMPA/KAR-EPSCs can be decreased only by decreasing glutamate release via presynaptic mechanisms. However, in fact, NMDA receptors have a much higher affinity for glutamate than do AMPA receptors and the concentration of glutamate achieved in the synaptic cleft may often be sufficient to activate NMDA, but not AMPA receptors (Kullmann and Asztely, 1998; Isaacson, 1999). We proposed that DA-induced depression of EPSCs is probably due to a decrease in presynaptic glutamate release and a simultaneous attenuation of the function of postsynaptic NMDA receptors. This proposal is consistent with recent findings in cultured striatal and hippocampal neurons (Lee et al., 2002; Lin et al., 2003). Lee et al. (2002) found that $D_1$ receptor agonist treatment significantly reduced the number of

NMDA receptors on the cell surface. When $D_1$ receptors are activated, NMDAR-EPSCs are inhibited by direct protein-protein interaction (Lee et al., 2002).

***The inhibitory effect of DA on EPSCs did not involve PKA and PKC pathways***

In our study, we observed that activation of AC by forskolin potentiated both NMDA and AMPA/KA receptor-mediated responses. This finding is consistent with observations from other investigating in the nAcb (Harvey and Lacey, 1996; Brundege and Williams, 2001). A study in the striatum also showed that NMDA responses were potentiated after stimulation of PKA (Blank et al., 1997; Colwell and Levine, 1995). However, we found that the inhibitory effect of DA on the EPSCs was not antagonized by the AC inhibitor, SQ 22536. In the presence of forskolin (10 μM), the ability of dopamine to depress synaptic transmission was not significantly altered (Harvey and Lacey, 1996). Although some studies report that PKA and PKC mediate the effect of DA (by $D_1$ receptor) on NMDA-evoked currents (Chergui and Lacey, 1999; Snyder et al., 1998), these findings on involvement of PKA or PKC are always related $D_1$ receptors enhancement of the currents evoked by NMDA agonists in the nAcb. We did not observe the enhancement action of DA on stimulus evoked NMDA currents nor found any involvement of PKA or PKC signal transduction pathway in the inhibitory effect of DA. In addition, the lack of change in EPSC decay time constants and no change in membrane current conductance of the postsynaptic cell while membrane potential was clamped between –100 and 40mV may indicate the effect of DA on EPSCs is independent of any effect on intrinsic properties of these neurons (Lee et al., 2002). Blocking the PKA signal transduction pathway by incubating slices in bath for 2-4 hours and in pipette with H89

(20μM) could not prevent the depression of EPSCs by DA, indicating the inhibitory effect of DA on EPSCs was not mediated via PKA route (Friedman et al., 1997; Deveney and Waddington, 1995; Wang et al., 1995). Also, Ro-32-0432, a PKC inhibitor failed to antagonize the inhibitory action of DA, excluding the possibility that PKC was involved in DA inhibitory process under our experimental conditions (Friedman et al., 1997; Lee et al., 2002). Consistent with our observations, $D_1$ and NMDA receptors exhibited an inhibition of NMDA receptor-mediated currents upon $D_1$ receptor activation by a $D_1$ receptor agonist in whole-cell patch-clamp recordings from hippocampal, striatal neurons, and HEK-239 cells coexpressing $D_1$ and NMDA receptors, has been demonstrated (Lee et al., 2002; Lin et al., 2003). The effect of $D_1$ receptor agonist could not be attributed to either PKC or PKA activation, and was independent of G-protein-mediated $D_1$ receptor signaling (Lee et al., 2002). This result suggests that $D_1$ receptors modulate NMDAR-EPSCs independent of PKA and/or PKC activation although numerous studies have demonstrated that NMDA receptor function may be regulated by G protein-coupled receptors, including $D_1$ receptors, through the activation of PKA/PKC dependent pathways (reviewed in Greengard, 2001).

Other possibilities could also explain why we found no involvement of PKA and PKC in the effects produced by DA. First, PKA and PKC may only be involved in the enhancement of the response induced by DA. Second, it is possible that some enzyme systems involved in the PKA and PKC pathways were washout by the whole-cell recording protocol. Third, in order to prevent voltage-sensitive sodium channels from generating action potentials, we applied QX314 in the recording pipette solution.

Blockade of sodium and potassium channels by QX314 may partially affect postsynaptic response. This appeared to be the case in cholinergic modulation of EPSCs (Zhang and Warren, 2002). Fourth, the inhibitory effect of DA on NMDAR-EPSC is possibly independent of the PKA and/or PKA system. We consider this the most probable mechanism for the inhibition of DA on NMDAR-EPSCs. More importantly, recent evidence provided by Lee and colleages (2002) shows that the $D_1$ modulation of NMDA receptors may be mediated through the regulation of NMDA receptor numbers on the cell surface, and that the $D_1$/NR2A coupling may play a role in the regulation of NMDA receptor expression on the cell surface. In addition, several previous findings also show that amphetamine inhibits the NMDA receptor-mediated responses by directly interacting with NMDA receptor/channel complex (Yeh et al., 2002) and a direct blockage of NMDA channels by DA (Castro et al, 1999).

The $D_1$-like receptor agonist SKF 38393, but not the $D_2$-like receptor agonist quinpirole, mimicked the action of DA. Consistent with these results, the $D_2$-like antagonist sulpiride failed to inhibit the depressive effect of DA and the $D_2$-like receptor antagonist/antiosychotic drug clozapine as well. However, the $D_1$-like receptor antagonist SCH 23390 could block the action of DA at a concentration of 10 μM, but not of 1 or 5 μM. This relatively high concentration of SCH 23390 needed to antagonize the action of DA may be explained by its competitive nature (Weiss et al., 1985). The concentration of DA used in this study may be related to the *in vitro* conditions of the experiment. The demonstrable effects, however, were obtained after 8-10 min of DA perfusion. In addition, rapid oxidation (Sutor and ten Bruggencate, 1990) as well as

uptake of DA may reduce the final concentration of DA at its site of action. In agreement with other studies in the nAcb (Chergui and Lacey, 1999) and studies in the parabrachial nucleus (Chen et al., 1999), we found that similar concentrations were necessary to elicit effects of DA on synaptic responses in *in vitro* conditions.

***Functional implications***

Our previous work on glutamatergic receptors-mediated EPSCs (Zhang and Warren, unpublished) and other studies in the distribution of $D_1$ receptors (Tarazi et al, 1999; Antonopoulos et al., 2002) have found that both NMDAR-EPSCs and $D_1$ receptor are predominantly expressed in the nAcb during early postnatal development. The synchronization of NMDAR-EPSCs and $D_1$ receptors during postnatal development may play a pivot role in neural plasticity (Pennartz et al., 1991, 1993; Kombian and Malenka, 1994; Schramm et al., 2002). As appetitive instrumental learning requires coincident activation of NMDA and $D_1$ receptors (Smith-Roe and Kelley, 2000) and $D_1$ receptor activation decreases the ethanol sensitivity of NMDA receptors in the nAcb (Maldve et al., 2002); $D_1$ and $D_5$ receptor activation is required for LTP (Kerr and Wickens, 2000) and $D_1$ receptor-dependent trafficking of NMDA receptors to postsynaptic sites in the neostriatum has also been found (Dunah and Standaert, 2001). These findings suggest that NMDA receptors and $D_1$ receptors are interactive, inter-regulated to maintain a balance (Cepeda and Levine, 1998; Lee et al., 2002). Hyperactivity of NMDA receptor-mediated function may result in an excitotoxicity due to $Ca^{2+}$ overload (Dawson et al., 1991; Chen et al., 1992; Ferreira, 1996; Aarts et al., 2002). On the other hand, an excessive DA may attenuate NMDA receptor-mediated function and so decreases $Ca^{2+}$

influx to the neurons during postnatal development, affecting plasticity. Recent hypotheses about the pathophysiology of schizophrenia have posited defects in excitatory amino acid transmission (e.g. Carlsson and Clarlsson, 1990; coyle, 1996; Olney and Farber, 1995; Do et al., 1995; Faustman et al., 1999; Tsai et al., 1995; reviewed by Meador-Woodruff and Healy, 2000). NMDA receptor antagonists produce effects in normal human subjects that resemble schizophrenic symptoms (Krystal et al., 1999) and drugs that alter glutamate transmission can ameliorate schizophrenic symptoms (Javitt et al., 1994; Tsai et al., 1998), suggesting defects in excitatory amino acid transmission. As schizophrenia is associated with a hyperfunction of the dopaminergic system (Gray et al., 1995; Joyce, 1993; Joyce and Meador-Woodruff, 1997), an enhanced DA-induced depression of the excitatory drive onto nAcb cells may cause imbalance in the related neurocircuits in earlier ages. Considering the fact that antagonists at DA receptors have a much higher antipsychotic potency, the nAcb may represent a potential site of such action. Just as schizophrenia often emerges during late adolescence or early adulthood, predominant NMDA receptors and DA receptors expressed in early postnatal development might play a pivotal role in the etiology of the schizophrenia.

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# CHAPTER V

# GENERAL DISCUSSION

## Experimental aim and results

Schizophrenia is a severe mental disease that could result from abnormalities in early brain development (Weinberger, 1995; Cannon, 1996; Woods, 1998; Isohanni et al., 2001; Remschmidt, 2002). The clinical syndrome produced by glutamatergic dysfunction, especially more pronounced NMDA receptor hypofunction, has been suggested as a possible substrate of the pathophysiology of schizophrenia (Carlsson and Carlsson, 1991; Meador-Woodruff and Healy, 2000; Newcomer and Krystal, 2001). In addition, discovering the distinct difference of physiological properties of the MS neurons during postnatal development from adulthood (Belleau and Warren, 2000) as well as an increased volume in the nAcb resulted from a neuronal death following prenatal cortical neurodevelopmental disturbances (Lauer et al., 2001), is the major motivation of the present work.

In the present study, we found that EPSCs evoked by local electrical stimulation in the nAcb had both AMPAR- (early) and NMDAR- (late) components. The late component increased to a maximum towards the end of the second postnatal week, and then decreased until it could be hardly detected after the third week. However, during pharmacological blocking of AMPAR-EPSCs, NMDAR-EPSCs could also be detectable even during fourth week with a faster decay time than during the early postnatal period. These results suggest that NMDAR-EPSCs are predominantly expressed during early postnatal development in the nAcb. Additionally, the ratio of amplitude of late to early component was decreased during the same period, which is in agreement with the finding that the expression of postsynaptic NMDA receptors is downregulated during postnatal

development (Bellingham et al., 1998; Futai et al., 2001; Isaacson and Walmsley, 1995). ACh decreased or increased glutamatergic transmission by respectively acting on muscarinic and nicotinic receptors probably located on excitatory terminals. Muscarinic depression of the EPSCs was mediated by M1/M4 receptors and nicotinic facilitation of the EPSCs was mecamylamine-sensitive. In addition, the inhibitory effect of ACh on NMDAR- but not on AMPAR-EPSC significantly increased during the first two postnatal weeks. Acting on D1 receptors, DA strongly inhibited NMDAR-EPSCs while moderately reducing AMPAR-EPSCs. The ratio of amplitude of the late component to the early components was significantly decreased, and the PPR was increased by DA at the hyperpolarized membrane potentials. Since the action of DA was resistant to the protein kinase inhibitors H89, Ro-32-0432 and the cAMP antagonist, QS22536, suggesting the inhibitory action of DA was independent of an action mediated by AC/PKA or PKC systems.

**Expression of NMDA receptor subunits and the performances of EPSCs**

Our results show that EPSCs evoked by local electrical stimulation in the nAcb have both AMPAR- and NMDAR-components. NMDAR-component increased to a maximum during the second postnatal week, and then decreased until it could hardly be detected after the third postnatal week. However, during blockade of the AMPAR-component, the NMDAR-component could be detected at later ages but with a faster decay time than that during the early postnatal period.

Our findings indicate that NMDAR-EPSCs are predominantly expressed during postnatal development. The changes in NMDAR-EPSC kinetics in our observation is consistent with the findings in other brain areas (Joshi and Wang, 2002; Flint et al., 1997;

Fox, 1995) and appear matched to the gene expression of NMDA receptor subunit mRNA during postnatal development in the neostriatum (Lau et al., 2003), visual and auditory cortex as well as in the thalamus (Cao et al., 2000; Hsieh et al., 2002). During the development of glutamatergic transmission in the sensory cortex, the proportion of synapses with detectable AMPAR-current increases, and the decay kinetics of NMDAR-current become faster. The change in NMDA receptor kinetics is believed to result from a developmental switch in the subunit composition of NMDA receptors. The NR1 subunit combines with various NR2A-D subunits to produce receptor subtypes with different kinetics. Of these, NR2A subunit-containing NMDA receptors have the faster decay times, whereas NR2B subunit-containing receptors have the slower decay times. A general trend shows that the contribution of the NR2B subunit is decreasing during development, which is associated with an increasing contribution of NR2A subunit-containing NMDA receptors to synaptic current. A gradual replacement or supplementation of NR2B by NR2A during postnatal development has been implicated in the speeding of NMDAR-EPSC decay—a phenomenon often linked with the ability of neuronal circuits to exhibit experience-dependent synaptic plasticity (Constantine-Paton and Cline, 1998). We found that NMDAR-component increased to a maximum during the second postnatal week, and then decreased, suggesting a gradual increase in NR2B subunit-containing NMDA receptors until the end of the second postnatal week. These late EPSCs during the second postnatal week are supposed to be distinctly decreased if ifenprodil was used.

Following the second postnatal week, NMDAR-EPSCs could be detected with a faster decay time, suggesting that the proportion of NR2A subunits-containing NMDA

receptors increased and gradually replacing or supplementing NR2B subunits after the second postnatal week. The kinetics of NMDAR-EPSC became shorter gradually due to more insertion of NR2A subunits so that its peak overlapped with the peak of AMPAR-EPSC in the compound EPSCs. Unmasked by AMPA receptor antagonist, NMDAR-EPSC was re-detectable even in later postnatal week.

A decrease in NMDAR-EPSC decay time with maturity has been described in other regions of the CNS (Carmignoto and Vicini, 1992; Hestrin, 1992; Takahashi et al., 1996). This decrease appears to be correlated with the appearance of NR2A subunits (Flint et al., 1997; Takahashi et al., 1996), and the expression of NR2A is increased by activity (Quinlan et al., 1999; Vallano et al., 1996). Thus, during postnatal development, there is an increased proportion of the NR2A subunit-containing NMDA receptor accompanied by a decreased proportion of NR2B (Yoshii et al., 2003). It has been further shown that the mature kinetics (decay time constant, 100ms) are characteristic of NMDA receptors composed of NR1 and NR2A subunits, whereas the immature form (350ms) is the characteristic of the receptors with NR1 and NR2B subunits (Williams et al., 1993).

**NMDA receptor and synapsogenesis**

We found in the morphological study that MS neurons are essentially aspiny from P0 to P10, and possess varicose dendrites, whereas they assume an adult spiny appearance only toward the end of the third postnatal week. These changes are concomitant with the evolution of NMDAR-EPSCs during the development.

$Ca^{2+}$ influx through glutamate receptors is thought to play a critical role in neuronal synaptogenesis and in the formation of neuronal circuitry during early

development (McDonald and Johnston, 1990). The NMDA receptor has been proposed to be the first glutamatergic receptor to appear during synaptogenesis (Baba et al., 2000; Durand et al., 1996; Isaac et al., 1997; Petralia et al., 1999). A large body of evidence has shown that disturbing the function of the NMDA channel during development can severely disrupt the wiring of defined neural circuits. It can also disrupt the developmental upregulation of the receptor protein itself and possibly many other molecular components of the synapse (Constantine-Paton, 1994). Studies further demonstrated that NMDA receptor mediates control of protein synthesis at developing synapses (Scheetz et al., 2000). Activation of NMDA receptors increases the translation of α $Ca^{2+}$/calmodulin dependent kinase II and the phosphorylation of eukaryotic elongation factor II during activity-dependent synaptic changes (Scheetz et al., 2000). Activation of the NMDA receptor also plays a role in structural plasticity. Studies on the development of axonal and dendritic arbors in the developing tectum also showed that early synaptic activity mediated by NMDA receptors promotes the growth of dendritic arbor in *xenopus laevis* tadpoles (Rajan and Cline, 1998), facilitating the formation of additional synaptic contacts (Cline, 2001). The NMDA receptor antagonist suppressed the transient increase in dendritic arbor at the beginning of the second postnatal week in the supraoptic nucleus neurons of rats (Chevaleyre et al., 2002). Conversely, application of NMDA increased dendritic branches in slices of P3-P6 rats. Their effects were inhibited by blockade of electrical activity, voltage-gated $Ca^{2+}$ channels, or intracellular $Ca^{2+}$ mobilization (Chevaleyre et al., 2002). Exposure of the developing brain during the period of synaptogenesis to anesthetic agents that block NMDA receptors can trigger widespread apoptotic neurodegeneration (Jevtovic-Todorovic et al., 2003). On the other

hand, chronic blockade of NMDA receptors in hippocampal slice cultures during early postnatal development results in a lower threshold for the induction of LTP (Savicc et al., 2003) and leads to a substantial increase in synapse number and results in a more complex dendritic arborization of CA1 pyramidal cells (Luthi et al., 2001). Consistently, chronic blockade of NMDA receptors delays the maturation of NMDA currents and increases the sprouting capacity of ipsilateral retinocollicular axons without disrupting their early segregation (Colonnese et al., 2001; Colonnese et al., 2003). These opposing results suggest that NMDA receptors may also be critical for the shaping and refinement of the structural and functional properties of neuronal circuits during early postnatal development (Luthi et al., 2001).

Glutamate promotes proliferation of striatal neuronal progenitors by an NMDAR-mechanism. Low concentrations of NMDA increased proliferation, whereas high concentrations were toxic (Luk et al., 2003). Moreover, nicotine sensitization increases dendritic length and spine density in the nAcb (Brown and Kolb, 2001), suggesting it plays an important role in the nAcb during postnatal development.

**Paired-pulse ratio and presynaptic mechanisms**

The paired-pulse protocol (PPP) has been widely applied to the identity of presynaptic mechanisms (Zucker, 1989, 1999; Kamiya and Zucker, 1994). It was used in our project to distinguish pre or postsynaptic sites at which ACh and DA could modulate EPSCs. The amplitudes of two EPSCs evoked by the PPP differ depending mainly on release probability of transmitters including the synaptic state during the moment of the arrival of the second spike (Dittman et al., 2000; Saviane et al., 2002). In general, the smaller amplitude is the probability of release to the first pulse, the more facilitated

amplitude is the response to the second pulse. This phenomenon is known as paired-pulse facilitation (PPF). In contrast, if the amplitude of the second response is depressed or smaller than the first one, it is called the paired-pulse depression (PPD) (Steven and Wesseling, 1998). Studies have shown that the probability of release depends on both residual $Ca^{2+}$ and the size of the available vesicles pool. The interplay between these two factors at the moment of arrival of the second spike would determine the direction of the paired-pulse modulation, i.e. PPF or PPD. The PPF observed in the majority of neurons in stationary conditions is accounted for by the residual $Ca^{2+}$ hypothesis, according to which the small fraction of $Ca^{2+}$ entering the terminal during the first spike increases the probability of transmitter release to a second action potential (Zucker, 1989) when vesicles pool is in constant. The PPD is presented at an entry of release sites into refractory states to depress transmitter release (Dittman et al., 2000; Dobrunz et al., 1997; Steven and Wesseling, 1998). This could be due to either the depletion of the readily releasable transmitter pool (Steven and Wesseling, 1998) or to residual $Ca^{2+}$ following the first stimulus depressing the $Ca^{2+}$ influx produced by the second stimulus (Kirischuk et al., 2002). Or both could occur after sustained depolarization of the presynaptic terminal (Wu and Borst, 1999; Dittman and Regehr, 1998). Obviously, both PPF and PPD are presynaptic phenomenonna (Zucker, 1989; Wu & Saggau, 1997; Dittman et al., 2000). Presynaptic inhibition of elicited transmitter release is mainly due to a reduction of presynaptic $Ca^{2+}$ influx through specific $Ca^{2+}$ channel types (Wu and Saggau, 1994, 1997). A reduction in release probability predicts an increase in the PPR that has shown to be enhanced when the extracellular $Ca^{2+}/Mg^{2+}$ ratio was lowered (Debanne et al., 1996; Canepari and Cherubini, 1998; Regehr and Stevens, 2001; Bellingham and Walmsley,

1999). In our experiments, both ACh and DA altered PPR in MS neurons, indicating the involvement of presynaptic mechanisms. Facilitation of PPR by DA suggests that DA acts mainly through a reduction of the presynaptic $Ca^{2+}$ influx (Wu and Saggau, 1997). The reduction in PPR produced by ACh can be attributed to either residual $Ca^{2+}$ binding to a molecular site intimately involved in regulating the probability of vesicle release or changes in size of the readily releasable pool of vesicles (Wu & Saggau, 1997; Dittman et al., 2000) or both factors. Additionally, depletion of a pool of readily releasable vesicles during repetitive presynaptic activity is a basic mechanism for the induction of short-term synaptic depression (Schneggenburger et al., 2002). Wu & Borst (1999) also found that the maintained synaptic transmission during tetanic stimulation is due to a rapid replenishment of reluctant vesicles into the releasable pool, the release of which is facilitated by the increase in residual $Ca^{2+}$ during the train. In fact, the average release probability of vesicles in the releasable pool is lower during synaptic depression. In addition, our finding on PPR changes of AMPAR- but not NMDAR-EPSCs probably suggests that the effect of DA on NMDAR-EPSCs involved postsynaptic sites, because presynaptic manipulations should produce parallel changes in AMPAR- and NMDAR-EPSCs (Poncer and Malinow, 2001).

**Direct interaction between NMDA receptor and D1 receptor**

Synaptic NMDA receptors play critical roles during brain development, plasticity, and pathology (Constantine-Paton and Cline, 1998; Dingledine et al., 1999; Zoghbi et al., 2000). The NMDA receptor is not a static resident of the synapse. Rather, the number and composition of synaptic NMDA receptors can be regulated (Wenthold et al., 2003), suggesting that a rapid regulation of postsynaptic NMDA receptors is one of the

mechanisms underlying developmental plasticity in the brain. In the study of the modulation of DA on NMDAR-EPSCs, we observed the ratio of amplitude of the late to the early component of EPSCs was decreased by the presence of DA. This suggests that the inhibitory action of DA on NMDAR-EPSCs was at least partially mediated by postsynaptic mechanisms. Since synaptic transmission affected from purely presynaptic mechanisms should have produced parallel changes in AMPAR- and NMDAR-EPSCs (Poncer and Malinow, 2001; Von Gersdorff et al., 1997; Tong and Jahr, 1994; Perkel and Nicoll, 1993). One possibility is that postsynaptic effects of DA on D1 receptor are voltage-dependent and are scarcely effective at –100mV, but at –40mV. Another possibility is that DA causes a decrease in the number of NMDA receptors. A downregulation in the number of NMDA receptors produced by DA has been demonstrated in MS cells in the striatum (Lee et al., 2002). Indeed, D1 receptors co-localized with NMDA receptors in the postsynaptic density (PSD) in the striatum suggest a direct interaction between D1 and NMDA receptors (Salter, 2003; Lee et al., 2002; Scott et al., 2002; Barria and Malinow, 2002; Fiorentini et al., 2003). In particular, that D1 receptor is apparently associated with both the NR1 and NR2A subunits, but not with the NR2B subunit (Fiorentini et al., 2003). The $D_1$ receptor does not interact with NR2B subunits in striatal PSD, probably explaining the postsynaptic effect of DA without obviously affecting the kinetics of NMDAR-EPSCs in our results.

Several lines of evidence indicate that different levels of synaptic NMDA receptor activation with corresponding degrees of $Ca^{2+}$ influx can lead to multiple effects. Low level of NMDA receptor activation produces synaptic depression, while higher levels of activation produce synaptic potentiation (Cummings et al., 1996; Zucker, 1999). Even

higher levels of activation can lead to cell death (Choi, 1995). Thus, the number and properties of NMDA receptors at a synapse must be under optimal control in order to allow the appropriate amount of $Ca^{2+}$ entry. Therefore, the interaction between NMDA and D1 receptors in MS neurons could be one of the mechanisms directing the trafficking of D1 and NMDA receptors to specific subcellular compartments. Recent observation shows that NMDA and D1 receptors partially overlap in the subcellular distribution and are assembled as oligomeric units in the endoplasmic reticulum and transported to the cell surface as a preformed complex (Fiorentini et al., 2003). Delivery of both D1 and NMDA receptors to the synapses is dependent on glutamate transmission (Barria and Malinow, 2002; Scott et al., 2002), suggesting that direct protein-protein interactions might direct the trafficking of these receptors to the same subcellular domain. Activation of D1 receptor decreased the number of NMDA receptors, which results in the inhibition of EPSCs (Lee et al., 2002). In contrast, activating NMDA receptors also alters the balance between D1 and D2 receptor signaling recruiting D1 receptors to the plasma membrane and spines without effecting on the distribution of D2 receptors (Scott et al., 2002). A study has shown the NR2A mRNA levels exhibited significant increases in DA deficiency postnatal mice in the nAcb (Fragioudaki et al., 2003), indicating that DA exhibits an inhibitory control on the expression of NMDA receptor subunits.

**NMDA receptor trafficking and use-dependent expression**

To remove or insert certain receptors or partial components of the receptors in accordance with the requirement is one of the tasks of receptor trafficking. The gradual replacement of NR2B subunits by NR2A during postnatal development is a typical use-

dependent expression of regulation. Synaptic trafficking of NMDA receptors is tightly regulated by synaptic activity and has subunit-specific rules with functionally important consequences (Barria and Malinow, 2002). For example, astrocytes express functional NR1 and NR2B after anoxia *in vitro* or ischemia *in vivo* (Krebs et al., 2003).

LTP and LTD strongly depend on NMDA receptor activity and are developmentally regulated in the nAcb (Pennartz et al., 1993; Kombian and Malenka, 1994; Schramm et al., 2002). However, LTP promotes a rapid surface expression of NMDA receptors in a PKC- and Src-family-dependent manner. Insertion of NMDA receptors may be a key step in regulating synaptic plasticity (Grosshans et al., 2002). It was reported that activation of mGluR1 regulates NMDA receptor trafficking by two opposing processes, i.e. either activation of mGluR accelerates NMDA receptor trafficking, resulting in the insertion of NMDA receptors and increasing NR1 surface expression (Lan et al., 2001) or activation of mGluR stimulates dendritic protein synthesis and LTD, leading to a rapid internalization of NMDA receptors from synapses in the hippocampus (Snyder et al., 2001).

**Interaction of transmitters in the nucleus accumbens**

In the nAcb, DA and glutamate, which come from extrinsic sources, as well as ACh and GABA from local circuit neurons, are all capable of influencing the activity of accumbal MS neurons. In the core region, the inputs onto spines and distal dendrites generally arise from extrinsic sources, whereas the synapses situated more proximally on dendrites or perikarya come from intrinsic sources (Meredith, 1999). The most common sites of convergence for corticoaccumbal and dopaminergic terminals are dendritic shafts

and spines of MS neurons with a distance between terminals of less than 2 microns (Kotter, 1994). Glutamate transmission is inhibited by the activation of D1 or M1 receptors and is potentiated by the activation of nicotinic receptors in the nAcb (Kalivas and Duffy, 1997; Charara and Grace, 2003; Kombian et al., 2003; Chergui and Lacey, 1999; Harvey and Lacey, 1997; Harvey and Lacey, 1996; Beurrier and Malenka, 2002; Nicola and Malenka, 1998; Nicola et al., 1996; Pennartz et al., 1992; Thomas et al., 2001; Zhang and Warren, 2002; de Rover et al., 2002; Sugita et al., 1991; Pennartz and Lopes da Silva, 1994). The inhibitory effects of ACh on NMDAR- but not on AMPAR-EPSC significantly increased during the first two postnatal weeks in our observation. This result is parallel to the expression of NMDAR-EPSCs as well as to the appearance of dendritic spines during early postnatal development. Possibly, the predominant expression of NMDA receptors is capable of leading to a positive feedback of ACh release or promotes the expression of muscarinic receptors in the nAcb. Other studies have shown that the activation of NMDA receptors induced the release of ACh in the nAcb and striatum (Jones et al., 1987; Buchholzer and Klein, 2002) as well as in the pontine reticular formation (Lydic and Baghdoyan, 2002).

In addition to the interaction between NMDA and ACh, other interactions will be helpful for us to understand nAcb sufficiently. Studies have shown that the cholinergic cell ablation enhances long-lasting behavioral changes in cocaine addiction whereas ACh enhancement prevented addictive behaviors to cocaine and morphine (Hikida et al., 2001, 2003). Systemic morphine decreases extracellular ACh and increases DA, indicating that ACh and DA act oppositely and that cholinergic activity could be under inhibitory DA control in the nAcb (Ressell et al., 1989; Rada et al., 1991; Wedzony et al., 1988).

Systemic administration of amphetamine leaded to an increase in extracellular ACh and DA levels while the concentration of GABA gradually decreased (Lindefors et al., 1992; Arnold et al., 2000). Further study demonstrated that amphetamine administration in the nAcb induced a bidirectional change in ACh release that was dependent on dose and opposing effects of nAcb D1 and D2 activation. Comparatively, low doses of amphetamine caused a D1 receptor-dependent increase in ACh release whereas higher doses of amphetamine resulted in a D2 receptor-mediated decrease (Keys and Mark, 1998). In contrast, the activation of muscarinic M4 receptors exerted a direct inhibitory control on D1 receptor signaling (Onali and Olianas, 2002), and stimulation of M1/4 also inhibited amphetamine-induced DA release in the nAcb (Ichikawa, 2002).

Several studies have shown that the activation of nicotinic receptors increase accumbal DA overflow in rats (Imperato et al., 1986; Di and Imperato, 1988; Johnson et al., 2000) whereas mecamylamine decreased accumbal DA output in mice treated chronically with nicotine (Gaddnas et al., 2002). Interestingly, nicotine-induced DA release was not only abolished by antagonist of nicotinic receptors in the nAcb (Nisell et al., 1994; Fu et al., 2000) but could also be attenuated either by the administration of NMDA or nicotinic receptors antagonists into the VTA (Schilstrom et al., 2000; Schilstrom et al., 1998; Fu et al., 2000). DA response to a relatively low dose of nicotine depends on the tonic activation of NMDA receptors in the VTA (Fu et al., 2000). Additionally, DA release resulting from NMDA receptor activation in the nAcb of rats is partially mediated by NO (Ohno et al., 1995). Interestingly, recent studies have also found that stimulating D1 receptors increases GluR1 phosphorylation and GluR1 surface expression in postnatal nAcb MS neurons in culture (Chao et al., 2002a, b).

**NMDA receptors and shizophrenia**

Schizophrenia is a chronic, severely disabling brain disorder with symptomatic onset in early adulthood. Hyperdopaminergic and hypoglutamatergic mechanisms are presently two basic hypotheses of schizophrenia (Zoghbi et al., 2000). Postmortem studies have revealed variable alterations in glutamate receptors and their modulators in schizophrenia. Several clinical trials indicate that agents enhancing NMDA receptor function via the glycine modulatory site reduce negative symptoms and can improve cognitive function in schizophrenics (Tsai and Coyle, 2002). Evidence implicating dysfunction of glutamatergic neurotransmission rests largely on the finding that antagonists of the NMDA receptor, especially the dissociative anesthetics like phencyclidine and ketamine, reproduce the cardinal symptomatic features of schizophrenia as well as the physiological manifestation of schizophrenia such as hypofrontality, impaired prepulse inhibition and enhanced subcortical DA release (Tsai and Coyle, 2002; Coyle et al., 2002; Marino and Conn, 2002; Millan, 2002; Greene, 2001). Recent studies have shown that NMDA receptor subunits (NR1, NR2B and NR2C) and PSD proteins are abnormally expressed in some cortical regions in schizophrenia and in the thalamic nuclei having reciprocal connection with limbic regions that have been implicated in schizophrenia (Meador-Woodruff and Healy, 2000; Hisham et al., 2000; Clinton et al., 2003), suggesting that glutamatergic dysfunction may occur not only at the level of receptor expression but also within intracellular signal transduction pathways associated with NMDA receptor (Clinton et al., 2003; Kajimoto et al., 2003). It has been suggested that early neonatal blockade of NMDA receptors in intact animals leads to the developmental disturbances to situational perception and

assessment of incoming sensory information (Latysheva and Raevskii, 2003; Millan, 2002; Ikonomidou et al., 1999; Deutsch et al., 1998). An endogenous peptide, N-acetyl-L-aspartyl-L-glutamate, has been found to antagonize NMDA receptors in a manner similar to known psychotogenic agents like ketamine or phencyclidine (Greene, 2001). Selective blockade of NMDA-dependent LTP of the recurrent inhibitory circuit may disrupt particular aspects of information processing involving learning and/or memory, consistent with the generation of abnormal associations in the hippocampus (Greene, 2001).

Cholinergic functions appear also abnormal in schizophrenia. Muscarinic receptor availability was significantly less, and positive symptoms of schizophrenia are correlated negatively with muscarinic receptor availability in the striatum and frontal cortex, indicating that the muscarinic system that modulates the NMDA receptor could be involved in the pathophysiology of schizophrenia (Coppola and Weinberger, 2003). Further, the presence of abnormal expression and function of the neuronal nicotinic receptor gene family in schizophrenia has been also reported (Leonard et al., 1998; Simosky et al., 2002).

The DA hypothesis of schizophrenia has been fruitful in producing a deeper understanding of pathophysiology of schizophrenia. In CA1 pyramidal neurons, activation of D2 receptors depressed excitatory transmission mediated by NMDA receptors. This depression resulted from the quinpirole-induced release of intracellular $Ca^{2+}$ and enhanced $Ca^{2+}$-dependent inactivation of NMDA receptors. The DA receptor-mediated depression was dependent on the "transactivation" of platelet-derived growth factor receptor β. Therefore, receptor tyrosine kinases transactivation provides a novel

mechanism of communication between dopaminergic and glutamatergic systems and might help to explain how reciprocal changes in these systems could be linked to the deficits in cognition, memory, and attention observed in schizophrenia and attention deficit hyperactivity disorder (Kotecha et al., 2002). Additionally, the finding of DA-NMDA receptor complex and the direct interaction between DA and NMDA receptors in limbic regions will probably be an important progress in the study of schizophrenia (Lee et al., 2002; Scott et al., 2002; Fiorentini et al., 2003).

Figure 1

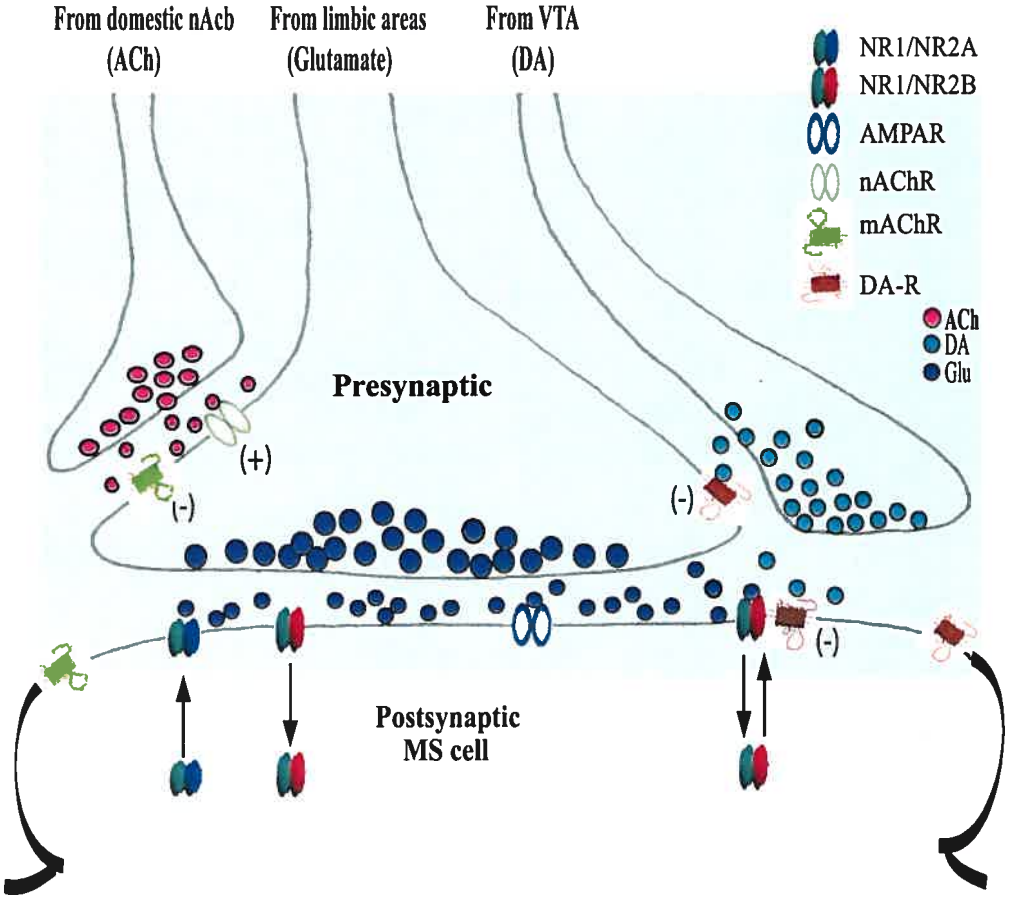


Fig. 1. Schematics showing the synaptic organization of the nAcb and a proposed mechanism of action of DA to decrease evoked EPSCs and ACh to decrease or increase EPSCs in the nAcb. The nAcb receives excitatory inputs from limbic areas, e.g., prefrontal cortex, amygdala, and hippocampus. It also receives a dopaminergic input from the ventral tegmental area (VTA). Cholinergic neurons are domestic in nAcb. Schematic also showing a proposed mechanism of change of NMDA receptor subunit components during the postnatal development and while DA receptors are activated in the nAcb. Arrows indicate the trafficking directions of NMDA receptor subunits (expression on membrane surface or internalization). (+): potentiation; (-): depression.

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