Dopaminergic Signaling in Development: Regulated for Mediating Biological Functions

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Biogenic amine neurotransmitters are implicated in a wide range of behavioral, cognitive, and homeostatic functions in the mature central nervous system (CNS). Biogenic amines include the catecholamines, dopamine, norepinephrine and epinephrine, as well as acetylcholine and serotonin. Each of these transmitter systems has a unique spatial and temporal pattern of onset during CNS development that has been characterized primarily in rodent models. It is important to note that these neuromodulatory systems appear early during embryogenesis, much earlier than the onset of synaptogenesis, suggesting that they also play important roles in brain development and formation of complex neural circuitry. It is therefore not surprising that alterations to these systems, either by pharmacological agents that affect synthesis or binding in the mature system, or developmentally due to toxic insults or genetic modifications, will have important consequences on brain function. In this review, I will focus on the developmental role of dopaminergic signaling although there are obviously important developmental milestones modulated through other transmitter systems. Regulation of dopaminergic signaling during development is important because current evidence suggests that dopamine (DA) exerts influence during specific sensitive periods in embryogenesis to mediate developmental processes including neuronal process extension. Also, dysregulation of dopaminergic neurotransmission in the striatum and cortex appears to contribute to many neurological and psychiatric disorders, including schizophrenia, Parkinson's disease, attention-deficit hyperactivity disorder, and drug addiction[1-7]. Developmental abnormalities in circuit formation and connectivity may contribute to these disorders even though clinical phenotypes usually become apparent only later in life.

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DOPAMINE IN THE ADULT CNS

The developmental functions mediated by dopaminergic signaling are not currently fully appreciated, however, more is known about the influences of DA in the mature brain. Synthesis of DA involves conversion of the amino acid L-tyrosine into L-dopa by the rate-limiting enzyme tyrosine hydroxylase (TH). Subsequent activity of DOPA decarboxylase results in final conversion to DA. DA is widely distributed in the CNS with important projections into the forebrain. The nigrostriatal tract consists of DA neurons with cell bodies located in the substantia nigra (SN) pars compacta and axonal processes terminating in the dorsal striatum. The striatum is a component of the extrapyramidal motor system and plays an essential role in the coordination of locomotor activity. The mesocorticolimbic pathway is another major forebrain dopaminergic projection. This pathway arises in the midbrain ventral tegmental area (VTA) and provides input to the nucleus accumbens, and some limbic regions including limbic cortical regions (medial prefrontal (mPFC) and anterior cingulate cortex (ACC)). These pathways are those important for mediating behaviors associated with motivation, reward (endogenous systems and drug abuse) and reinforcement, as well as cognitive and executive functions including attention[8-9].

ONTOGENY OF DOPAMINERGIC NEURONS

Detection of TH immunoreactivity has proven to be a useful method for identifying DA neurons during development. Although initial studies were conducted in rodents, parallels have been drawn between other models with available data on rabbits, non-human primates and humans. In the rat midbrain, TH is first apparent at Embryonic day (E)12-13 of an approximate 21 day gestation, and by E14 of an approximate 30 day gestation in the rabbit. Midbrain DA neurons are produced between E36 and E43 of a 165 day gestational period in the monkey[10] and appear during the second month of gestation in humans[11]. Thus in all species examined, dopaminergic neurons are detected very early in development, consistent with a morphogenic role of DA. After initial appearance in the midbrain,

dopaminergic axons project rostrally to target regions in the forebrain. DA axons can be detected in the cortex a few days later[12]. Dopaminergic input is thus already present in the cortex while pyramidal neurons are reaching their laminar positions in the more superficial layers, further support for a morphogenic role of DA. Limbic cortical regions, such as the ACC and mPFC, together referred to as the medial frontal cortex (MFC), receive the densest dopaminergic innervation. The density of TH-positive axons in the cortex increases gradually over development then declines postnatally to reach adult levels during puberty. This protracted postnatal increase in DA content occurs over a time period during which a number of developmental milestones occur that may involve transmitter signaling, such as synaptic maturation and obtaining competency on working memory tasks[13].

DOPAMINERGIC SIGNALING: REGULATION AT THE LEVEL OF RECEPTORS

DA interacts with specific receptor proteins on neuronal membranes to modulate the acute responsiveness of the cell to other synaptic inputs. DA also mediates longer-lasting effects through induction of nuclear changes in gene expression and synaptic plasticity[14]. DA receptors are guanine nucleotide binding protein (G-protein) coupled receptors (GPCRs) characterized by an extracellular N-terminus, intracellular C-terminus and seven membrane spanning segments. The receptors interact through the third intracellular loop with specific G-proteins to induce intracellular second messenger signaling cascades including regulation of calcium and potassium channels on the postsynaptic cell[15-16]. There is also auto-regulatory influence of DA through presynaptic receptor activation. Transmitter action is terminated by re-uptake into the presynaptic terminal by a high affinity plasma membrane dopamine transporter (DAT) in the peri-synaptic area and enzymatic degradation by monoamine oxidase (MAO) or catechol-*o*-methyl transferase (COMT).

Receptors sensitive to DA are divided into two classes based on their pharmacological profiles, sequence homology and signal transduction systems. D_1-like receptors, including the D_1 and D_5 receptor subtypes, couple to the stimulatory $G_{\alpha s}$ protein, of which there exists a long and short isoform, to increase activity of adenylate cyclase (AC) to synthesize cyclic adenosine monophosphate (cAMP). D_2-like receptors, including the D_2, D_3, and D_4 receptor subtypes, have antagonistic functions to D_1-like receptors and couple to $G_{\alpha i/o}$ to inhibit synthesis of cAMP[15-17]. Developmentally, receptor transcripts can be detected in the dorsal striatum and cortex by E14 in the rat and by E12 in the mouse[18-19]. In the monkey, DA receptors appear in target regions of DA input by the first quarter of gestation[20-21] and in humans, DA receptor binding sites have been detected by the twelfth week of gestation[22]. Receptor expression increases throughout prenatal and early postnatal development to reach adult levels of expression between Postnatal day (P)14 and P21 in rodents[23-26]. DA receptors are thus also present early in development, still consistent with a role for DA in mediating circuit formation.

Each receptor subtype possesses distinct cellular and/or regional distributions and pharmacological profile. In the dorsal striatum, dopaminergic signaling is mediated primarily through the D_1 receptor (D_1R) and the D_2 receptor (D_2R) which are expressed in greater abundance than the other subtypes and enriched in specific populations of efferent GABAergic medium spiny neurons, although there is evidence of co-localized populations. Neurons projecting from the striatum to the SN pars reticulata are enriched in the D_1R and co-express the peptide substance P. These neurons are involved in the direct extrapyramidal pathway. Neurons projecting to the external globus pallidus, on the other hand, are enriched in the D_2R and co-express enkephalins. These neurons are involved in mediating the indirect pathway. Receptor protein expression is high in the SN pars reticulata and external globus pallidus, but no receptor mRNA has been detected in these regions indicating that the receptors are present on the axonal process associated with the projection neurons from the striatum[15-16].

DOPAMINERGIC SIGNALING: REGULATION AT THE LEVEL OF G-PROTEINS

G-proteins serve as signal transducers between membrane-bound receptors and internal cellular effector systems. G-proteins exist in hetero-trimeric complexes composed of a G_α subunit in association with $G_{\beta\gamma}$ which exists as a functional dimer. Activation of the G-protein complex is controlled by a regulatory cycle involving receptor-activated exchange of GDP for GTP on G_α, dissociation of the trimer, activation of effector molecules, and inactivation through GTPase activity of G_α. The regulatory protein $G_{\alpha s}$ is responsible for stimulatory G-protein signaling in most cell types, however the striatum contains low expression of $G_{\alpha s}$. In the striatum, D_1R signaling is complicated by the presence of another stimulatory G-protein, $G_{\alpha olf}$ which exists in greater abundance than $G_{\alpha s}$[27-28] and is classically known to be important in olfactory signal transduction. More recently, $G_{\alpha olf}$ has been shown to couple to D_1Rs in the striatum to increase activity of AC[29]. $G_{\alpha olf}$ shares more than 80% sequence homology with $G_{\alpha s}$ but may fall under different regulatory controls[30]. In the striatum, $G_{\alpha olf}$ is developmentally regulated; increasing in expression

from P0 to P14 in mice before reaching a plateau after P14[30]. This developmental trend in $G_{\alpha olf}$ expression is reflected in forskolin stimulated cAMP activity in AC assays in which cAMP signaling increases significantly from P0 to P14 and plateaus at ages thereafter. $G_{\alpha olf}$ may be an important mediator in DA signaling through D_1Rs in the striatum as indicated by the loss D_1R-stimulated cAMP production in $G_{\alpha olf}$ knock-out mice[29]. A blunted cocaine or selective D_1 agonist-induced locomotor response has also been observed in these mice indicating a role for this protein in transducing this DA-mediated response[27].

Apart from $G_{\alpha s}/G_{\alpha olf}$ coupling to activate AC in the striatum, DA receptors have also been implicated in intracellular calcium mobilization through coupling to $G_{\alpha q}$ and activation of phospholipase C[31]. In this pathway, inositol triphosphate (IP_3) and diacylglycerol (DAG) second messengers are generated from phosphatidylinositol (PI) metabolism. Liberated IP_3 then binds intracellular receptors to release calcium from intracellular stores. Activation of the PI hydrolysis pathway has been shown to be triggered by specific D_1-like agonists that can be inhibited by co-application of D_1–like antagonists or co-application of D_2–like antagonists. D_2R-like agonists alone, however, are not able to stimulate calcium release. Additionally, it has been observed that D_1–like agonists differentially stimulate PI hydrolysis and /or AC activity to varying degrees indicating that AC-coupled and PI hydrolysis-coupled D_1Rs are distinct molecular and pharmacological entities[32-34]. It has recently been demonstrated that hetero-oligomers containing D_1 and D_2 receptors associate in neurons and co-activation of the receptors rapidly activates the $G_{\alpha q}$ pathway triggering calcium release and activation of calcium dependent molecules such as CaMKIIα[35-37]. The D_1R/D_2R hetero-oligomeric activation of $G_{\alpha q}$ is distinct from D_1R and D_2R activation of $G_{\alpha s}/G_{\alpha olf}$ or $G_{\alpha i/o}$, respectively[37-38]. D_1R-$G_{\alpha q}$ coupling has also been observed in the cortex, amygdala and hippocampus[39].

DEVELOPMENTAL INSULTS ALTER DOPAMINERGIC SIGNALING

As has been thus far noted, dopaminergic signaling is developmentally regulated at the level of dopamine expression, receptor expression and expression of G-proteins. Evidence from our laboratory and others investigating the effects of prenatal cocaine exposure on brain development in rabbits indicates that prenatal cocaine during a sensitive period in development impairs signal transduction through D_1Rs in the striatum and cortex. Impaired D_1R signaling results in permanent abnormalities existing for the life-time of the offspring including aberrant process elongation in the ACC and altered responsiveness of neurons in culture after D_1R activation with selective D_1R-like agonists[40-41]. This model is just one example of how a pharmacological challenge to developing brain circuits results in long-lasting changes in signaling (D_1R signaling in this case) perhaps as a compensatory mechanism to adapt to the *in utero* environment. Pharmacologically, cocaine interacts with the high affinity transporters of DA, norepinephrine and serotonin. Cocaine binds to the transporters and effectively blocks re-uptake of these monoamines into the presynaptic nerve terminal. As a result, the extracellular concentration of neurotransmitters is increased thus prolonging receptor activation. This effect also occurs *in utero* when a fetus is exposed to cocaine prenatally as cocaine readily crosses the placental barrier to inhibit DA uptake.

Prenatal cocaine exposure results in abnormal regulation of dendritic growth of cortical neurons in regions receiving dense DA input, without like changes in cortical regions receiving dense input from other transmitter systems such as visual or somatosensory cortex which both contain high serotonin content[43,46]. Under normal developmental parameters, it has been shown that DA receptor activation produces opposing growth phenotypes dependent upon the receptors activated and the functional properties of these receptors in various brain regions. In response to the addition of a selective D_1-like agonist to an embryonic culture, neurons isolated from the MFC exhibit decreased spontaneous neurite outgrowth in a dose-dependent manner, while striatal neurons show increased process elongation. Conversely, selective D_2R activation promotes neuronal outgrowth in the cortex while inhibiting growth in the striatum[42-45]. Cultures isolated from the MFC of cocaine-exposed offspring exhibit greater spontaneous neurite outgrowth than neurons isolated from saline-exposed embryos. Cocaine-exposed cultures are also insensitive to the addition of exogenous D_1-like agonists to the culture indicating changes in responsiveness of the neurons to stimulation[43,46]. In coronal brain slices from cocaine-exposed progeny, permanent changes in the structure and trajectory of dopaminoceptive neurons in the ACC are visible. When quantified, a 40-50% increase in length of apical dendrites in layers III and V pyramidal neurons produce a characteristic "wavy" dendritic phenotype (**Figure 1a**)[40]. Increased length of dendrites is reflective of changes in local circuitry and loss of D_1R signaling which would normally serve to mediate inhibition of neuronal process development[43,46]. Similar structural abnormalities due to loss of D_1R signaling are exhibited in D_1R knock-out mice[47].

Investigating the underlying mechanisms contributing to diminished D_1R coupling following

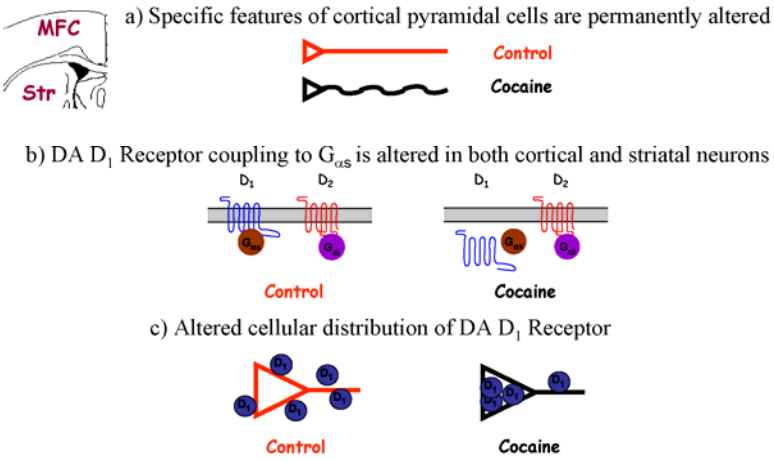

Figure 1 | **Prenatal cocaine exposure during a discrete period of embryogenesis results in long-lasting changes** in: **a** | cortical apical dendrite morphology, **b** | D_1R-$G_{\alpha s}$ coupling and **c** | D_1R subcellular distribution in cocaine exposed offspring.

prenatal cocaine exposure *in utero*, our laboratory established that there is a permanent reduction of DA-induced D_1R-$G_{\alpha s}$ coupling without a change in $G_{\alpha s}$ protein expression or total receptor density (**Figure 1b**). There is, however, a redistribution of the subcellular localization of the receptors such that fewer receptors are expressed on the plasma membrane (PM) while a larger proportion of receptors are permanently maintained within intraneuronal compartments[43]. Reduced surface expression of D_1Rs therefore reduces the number of receptors available for coupling to $G_{\alpha s}$ after agonist exposure (**Figure 1c**). The reduced coupling is specific for the D_1R - $G_{\alpha s}$ complex because coupling is not reduced for other $G_{\alpha s}$ coupled receptors, D_2R-$G_{\alpha i/o}$ coupling or muscarinic cholinergic receptors coupling to $G_{\alpha o}$ (in DA-rich areas)[46,48-49]. Disruptions in D_1R-$G_{\alpha olf}$ coupling have yet to be investigated. Alterations in signaling may be an adaptive response to the disrupted balance of DA and excessive receptor stimulation during development. Altered dopaminergic transmission has also been observed in other models of developmental modulation of DA content or receptor activation including dopamine depletion after denervation by 6-OHDA lesion[50] or TH inactivation[51], constitutive receptor activation in DAT knock-out mice[52-53], or selective modulation of signaling through D_1Rs or D_2Rs[54-55].

DOPAMINERGIC SIGNALING: REGULATION BY RECEPTOR AVAILABILITY

The availability of GPCRs at the PM is dynamically regulated by the neuronal environment including levels of neurotransmitter, intraneural trafficking and degradation. Modulation of the receptor density available for ligand binding is a key mechanism in the regulation of neuronal excitability and signaling. GPCRs are synthesized and folded in the endoplasmic reticulum (ER) before being transported to the Golgi apparatus where the proteins mature with addition of post-translational modifications. GPCRs are then targeted to the appropriate cellular membrane where they are able to interact with neurotransmitters[56-57]. The molecular mechanisms behind trafficking to the PM, receptor localization and surface expression are not fully understood, however, it is clear that D_1Rs exist as components of signaling complexes that can include channel proteins, other GPCRs, as well as scaffolding, cytoskeleton, and chaperone proteins[58-59]. DA receptor interacting proteins may be important regulators of D_1R transport from the ER and surface expression on the PM[60-61]. Specific post-translational modifications of the receptor are also implicated in regulating surface expression. Common modifications include glycosylation, palmitoylation, phosphorylation and ubiquitination.

N-linked glycosylation initiated in the ER and completed in the Golgi is the most common modification of GPCRs. Glycosylation involves the addition of oligosaccharides to specific asparagines residues with the consensus sequence NXS/T. The D_1R contains two consensus sites, one in the N-terminus region and the other in the second extracellular loop[16,62]. Receptor glycosylation might be important for D_1R PM localization and/or surface expression as it has been shown for other GPCRs[57], however, the data are conflicting regarding the role of D_1R glycosylation and PM expression[62-63]. D_1R glycosylation, however, is not necessary for ligand binding or coupling to G-proteins[63]. The D_1R has also been shown to undergo post-translational addition of fatty acid palmitate moieties at cysteine residues (Cys347 and Cys351) in the carboxyl tail of the receptor[64-65]. Palmitoylation of the receptor is likely involved in anchoring it to the membrane[16] as the majority of palmitoylated proteins are found at the PM[66]. As was shown with glycosylation of the receptor, it has also been shown that palmitoylation of D_1Rs is not involved in ligand binding or G-protein coupling[64]. Palmitoylation has also been shown not be involved with agonist-induced stimulation of AC or desensitization of D_1Rs[64].

Acute stimulation of receptors by DA reduces the number of receptors on the PM through a series of regulated processes, densensitization and internalization, for a period of time until removal of the ligand. Chronic stimulation, on the other hand, also reduces the number of receptors at the cell-surface but likely through different mechanisms of down-regulation. DA-mediated receptor activation promotes phosphorylation of the receptor at serine and threonine residues in the C-terminal region and third intracellular loop by receptor specific G-protein

coupled receptor kinases (GRKs) and cAMP-dependent kinases such as protein kinase A (PKA) activated by second messengers[67-70]. Phosphorylation of the receptor recruits binding of arrestin to the third cytoplasmic loop thus promoting uncoupling of the G-protein from the receptor. Arrestin targets the receptor to clatharin coated pits and recruits transport machinery for formation of the early endosome. Receptors are then internalized into intraneuronal compartments and de-phosphorylated by protein phosphatases before recycling back to the cell-surface in a resensitized state in which the receptors are competent to signal again. Alternatively, receptors can be trafficked to lysosomes or proteosomes for degradation[68-70]. Ubiquitination is an important modification made to receptors targeted for this pathway involving the covalent attachment of the small molecule, ubiquitin, to lysine residues of targeted proteins[71].

Alterations in post-translational modifications to DA D_1Rs are likely contributors to the underlying mechanisms behind the uncoupling of the receptor from $G_{\alpha s}$ and redistribution of the receptors after prenatal cocaine exposure. Changes in receptor modifications could potentially reduce delivery of receptors to the PM, thus keeping receptors sequestered in the ER or Golgi apparatus. Alternatively, aberrant modifications could increase the rate of receptor desensitization and internalization without proportional changes in resensitization. There is some evidence suggesting hyper-phosphorylation of receptors after prenatal cocaine exposure due to chronic receptor stimulation *in utero*. Receptor stimulation has been shown to increase phosphorylation of dopamine and cAMP-regulated phosphoprotein of 32kDa (DARPP-32) through activation of PKA. PKA phosphorylates DARRP-32 on Thr[34] thus converting it into an inhibitor of protein phosphatase 1 (PP1). PPI is responsible for de-phosphorylating many cellular substrates, thus decreased PP1 activity after cocaine exposure *in utero* could be responsible for maintaining receptors internally[72].

CONCLUSIONS

As has been reported, dopamine transmission modulates important events during development including neuronal process extension and establishment of normal circuitry. Insults to the dopaminergic system during development, such as chronic receptor stimulation *in utero*, result in permanent changes in dopaminergic signaling which may play a large role in the manifestation of neuropsychiatric disease states later in life. These alterations in signaling can result from the redistribution of receptors from the PM to internal compartments where they are no longer able to couple to G-proteins and mediate a response in the presence of ligand. Dissection of the molecular mechanisms behind alterations in receptor availability and subsequent changes in signaling cascades is relevant to understanding the pathophysiology behind diseases involving dysfunction of dopaminergic transmission whether it is hyper- or hypo-activity of the system. In fact, changes in dopamine receptor density have been observed in many diseased states. In schizophrenia, the density of D_2Rs has been shown to be elevated while the density of D_1Rs remains unchanged. In Parkinson's disease, increased D_1 and D_2 receptor densities has been shown to accompany loss of dopaminergic input into the midbrain. Similarly, loss of D_1 and D_2 receptor densities has also been observed in Huntington's disease patients. The studies proposed in the aims to follow are therefore not only important for understanding the normal and pathological states of the receptors but also in designing therapeutics for treating these disorders[73].

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This paper is highlighted because it clearly demonstrates the importance of D1R-Gaolf coupling and signaling in the striatum and the involvement of the protein in DA-mediated behaviors which were once largely thought to be mediated by D1R coupling to Gas. D1R-Gaolf coupling likely falls under different regulatory controls than D1R coupling to Gas thereby adding specificity to the signaling in the striatum. D1R coupling to G-proteins is likely to not only be regulated regionally, but developmentally as well.
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FURTHER INFORMATION

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