Published in final edited form as:

J Comp Neurol. 2006 September 20; 498(3): 415–430. doi:10.1002/cne.21034.

Plasticity within striatal direct pathway neurons following neonatal dopamine depletion is mediated through a novel functional coupling of serotonin 5-HT₂ receptors to the ERK 1/2 Map Kinase pathway

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Abstract

Dysfunction within the striatal direct and indirect projecting systems arises following 6-OHDA-induced dopamine depletion, highlighting the central regulatory function of dopamine in motor systems. However the striatal 5-HT innervation remains intact after 6-OHDA lesions, suggesting that the 5-HT system may contribute to the lesion-induced dysfunction, or alternatively, it may adapt and compensate for the dopamine deficit. Neonatal 6-OHDA lesions actually give rise to a 5-HT axonal hyperinnervation within the dorsal striatum, further reinforcing the idea that the 5-HT system plays a central role in striatal function following dopamine depletion. Here we show that neonatal but not adult 6-OHDA lesions result in a novel coupling of 5-HT₂ receptors to the ERK1/2/MAP Kinase pathway, a signaling cascade known to regulate neuronal plasticity. Chloroamphetamine-induced 5-HT release or direct stimulation of striatal 5-HT₂ receptors via the 5-HT₂ agonist DOI, produced robust ERK1/2 phosphorylation throughout the dorsal striatum of neonatal lesioned animals, a response not observed within the intact striatum. Pretreatment with the select 5-HT₂ receptor antagonist Ketanserin blocked DOI-induced ERK1/2 phosphorylation. This drug-induced ERK1/2 phosphorylation was subsequently shown to be restricted to direct pathway striatal neurons. Our data shows that adaptation of direct pathway neurons following neonatal 6-OHDA lesions involves coupling of 5-HT₂ receptors to the ERK1/2/MAP Kinase cascade, a pathway not typically active in these neurons. As dopamine mediated signaling is redundant following 6-OHDA lesions, 5-HT mediated stimulation of the ERK1/2/MAP Kinase pathway may provide an alternative signaling route allowing the regulation of neuronal gene expression and neuronal plasticity in the absence of dopamine.

Keywords

striatum; receptor coupling; serotonin

Introduction

The nigro-striatal dopaminergic input to the striatum plays a central role in regulating motor systems. 6-hydroxydopamine lesioning of this pathway produces several different behavioral models which have been used to examine the effects of dopamine depletion on striatal neurons and ultimately motor function. Lesions performed in adult rats result in slowed movements, modeling Parkinson's disease (Arbrous et al., 1992), whereas lesions in

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Associate Editor: Paul E. Sawchenko

neonatal rats resulted in hyperactivity, this time modeling Attention Deficit–Hyperactivity Disorder (Abrous et al., 1992; Penit-Soria et al., 1997; Reader and Dewar, 1999). Although 6-OHDA treatment leads to permanent dopamine ablation, the serotonergic projection to the striatum remains intact, and neonatal lesions actually lead to a 5-HT axonal hyperinnervation within the dorsal striatum.

Numerous studies have attempted to decipher the alterations in striatal neurotransmitter systems following neonatal lesions, examining both behavioral and neurochemical changes. Subsequent to neonatal 6-OHDA lesions, juvenile rats showed increased spontaneous motor hyperactivity, and hyperactivity following D1 dopamine receptor agonist treatment (Shaywitz et al., 1976a; Breese et al., 1984). Paradoxically, the hyperactivity was suppressed following amphetamine or methylphenidate treatment (Shaywitz et al., 1976b; Davids et al., 2002), an affect also seen in ADHD patients. Amphetamines not only release dopamine, they can also release serotonin (Rudnick and Wall, 1992; Levi and Raiteri, 1993; Wise, 1996; Crespi et al., 1997; Koob et al., 1998). Ensuing experiments showed that the striatal 5-HT system was fundamentally involved in regulating motor function in neonatal 6-OHDA lesioned animals (Heffner and Seiden, 1982; Bishop et al., 2003, 2004).

Several unique anatomical changes occur following neonatal 6-OHDA lesions, which do not arise following adult 6-OHDA lesions. Firstly, 5-HT axons in the dopamine depleted striatum sprout forming a 5-HT axonal hyperinnervation (Stachowiak et al., 1984; Blue and Molliver, 1987; Towle et al., 1989). Secondly, 5-HT_{2A} receptor expression increases significantly on direct pathway neurons (Laprade et al., 1996; Basura and Walker, 1999), even though 5-HT_{2A} receptors are expressed on both direct and indirect striatal projecting neurons (Ward and Dorsa, 1996).

Neonatal lesions also result in altered expression of preprotachykinin (decrease) and preproenkephalin (increase) in direct and indirect striatal pathway neurons respectively (Sivam et al., 1987). Chloroamphetamine induced 5-HT release (Basura and Walker, 2000) or 5-HT₂ receptor activation via DOI (Basura and Walker, 2001; Campbell et al., 2001; Campbell and Walker, 2002), increased preprotachykinin mRNA expression to control values, but did not affect preproenkephalin mRNA expression. This suggested that 5-HT acting via 5-HT₂ receptors could regulate preprotachykinin expression selectively in direct pathway neurons. Taken together with the behavioral data, these studies demonstrated how 5-HT could regulate direct pathway neurons and ultimately motor function, following neonatal dopamine depletion.

The intracellular mechanisms that mediate the effects of 5-HT within the neonatal lesioned striatum are poorly understood. Mounting evidence has demonstrated a central role for the ERK1/2/MAP Kinase pathway in both neuronal and synaptic plasticity (Kornhauser and Greenberg, 1997; Impey et al., 1999; Sweatt, 2001; Sweatt, 2004; Thomas and Huganir, 2004). Recently we showed that following adult 6-OHDA lesions, supersensitivity within direct pathway striatal neurons results from a novel coupling of D1 dopamine receptors to the ERK1/2 MAP Kinase pathway (Gerfen et al., 2002). With this in mind, we examined the effects of neonatal and adult 6-OHDA lesions on 5-HT systems within the striatum and examined whether ERK1/2/MAP Kinase is involved in the adaptations.

Materials and Methods

Animal treatments

Nigrostriatal dopamine lesions—All animal procedures used in this study were in accordance with the NIH guide for the care and use of laboratory animals. Two distinct animal models were used in these studies, in one lesions were made in the adult, and in the

other, lesions were made in the neonate. Both involved unilateral lesions of the nigrostriatal dopamine system with the neurotoxin, 6-hydroxydopamine (6-OHDA). Sprague Dawley rats (Taconic Farms, Germantown, N.Y.) were used in all experiments. For the adult lesions, rats approximately 300g in weight were anaesthetized with sodium pentobarbital (67mg/kg), placed in a stereotaxic frame and a unilateral lesion was produced by infusing 6-OHDA (16µg in 2µl) into the substantia nigra. Animals were returned to their home cages for three weeks with unrestricted access to food and water. For the neonatal lesions, postnatal day 3 (P3) rat pups were cooled on ice and unilateral lesions were produced by injecting 2µl (16µg) of 6-OHDA (Sigma, St. Louis, MO) into the right striatum (Penit-Soria et al., 1997). Animals were warmed on a heating pad and returned to the dam in the home cage. Animals were weaned on P21 and housed in groups of 2–3 until they were at least 60 days old.

Drug treatments—Following 60days (for neonatal lesions) or three weeks (adult lesions), animals were treated with a single or a combination of pharmacologic agents. Groups of animals received vehicle, p-Chloroamphetamine (PCA) (1–20mg/kg, i.p.) (Regis Chemical Company, Morton Grove, IL), or DOI (1–10mg/kg, i.p.) (Sigma, St. Louis, MO) and were perfused 15 or 60mins after drug treatment. For the antagonist experiments animals were pretreated with vehicle or Ketanserin (1–40mg/kg, i.p.) (Sigma) 30mins prior to DOI or PCA, and perfused either 15 or 60mins after DOI treatment.

Histology

Following the appropriate treatment and time course, animals were deeply anaesthetized with sodium pentobarbital and transcardially perfused with 4% formaldehyde in phosphate buffer, pH 7.4. Brains were removed and post-fixed in the same formaldehyde solution overnight at 4°C. The brains were then transferred to a 20% sucrose solution (in PBS, pH 7.4) and were kept at 4°C until they sank to the bottom of the tube. At this point each brain was sectioned on a sliding microtome and a series of 40µm sections throughout the striatum were collected and stored at 4°C in PBS until processed.

A series of coronal sections from each brain were processed for immunohistochemistry. Free floating sections were incubated overnight in blocking solution (0.2% Triton X-100, 2% normal serum in PBS) containing primary antisera for TH (1:250, Pel-Freez Biologicals, Rogers, AK), SERT (1:10,000, Chemicon International, Temecula, CA), pERK1/2 (1:250, Cell Signaling Technology) or c-fos (1:4000, Genesys The Woodlands, TX). The primary antibodies were visualized using the Vectastain ABC elite protocol (Vector laboratories) and 3,3'-diaminobenzidine.

Antibody specificity—The pERK1/2 antibody (#9101) was generated in rabbits against a synthetic peptide (KLH-coupled) corresponding to residues around Thr202/Tyr204 of human p44 Map kinase. Western analysis (both manufacturers and our own data) gave two bands corresponding to p44 and p42 MAPK respectfully.

The SERT antibody used was a mouse anti-serotonin transporter monoclonal antibody (MAB 1564), raised against an N-terminus/GST fusion protein corresponding to amino acids 1–85. The specificity and co-localization of SERT staining with 5-HT immunoreactivity has previously been determined (Brown and Molliver, 2000).

The c-fos antibody (PC38) was raised in rabbit against a synthetic peptide (SGFNADYEASSRC) corresponding to amino acid residues 4–17 of human c-Fos. References concerning antibody applications and specificity are cited on the manufacturers' product sheet (Calbiochem; Anti-c-Fos (Ab-5) Rabbit pAB, Cat. No. PC38).

The TH antisera was generated in rabbits against SDS-denatured tyrosine hydroxylase (Pel-Freez, Rogers Arkansas. Code number: P40101). The specificity has been previously determined by western analysis and immunohistochemistry (see manufacturers data sheet).

Double labeling—Immunohistochemistry and non-radioactive *in situ* hybridization were combined to detect pERK1/2 or Fos, and enkephalin within striatal projecting neurons. All sections that were processed for double labeling studies were mounted onto gelatin coated slides and air dried. The slides were then transferred through the following solutions and again air dried: 4% formaldehyde in PBS (10min), 0.25% acetic anhydride in triethanolamine (0.1M, pH 8.0, 10min), 70% alcohol (2min), 95% alcohol (2min), 2× 100% alcohol (2min each), 2× 100% chloroform (5min each), 2× 100% alcohol (5min each) and 95% alcohol (2min).

For pERK1/2 – enkephalin double labeling, *in situ* hybridization buffer (50% formamide, 600mM NaCl, 80mM Tris HCl, pH 7.5, 4mM EDTA, 0.1% sodium pyrophosphate, 0.2% SDS, 2% sodium polyacrylate, 100mM dithiothreitol, 1μg of tRNA, 1μg total RNA and 0.4μg of salmon sperm DNA) containing a digoxigenin-labeled ribonucleotide probe complementary to the enkephalin mRNA sequence was applied to the slide mounted sections, the sections were covered with a glass cover slip and then incubated overnight at 55°C in a humid chamber. Following RNase A treatment for 30min (20mg/ml), slides were washed four times (20min each) in 0.2× SSC at 65°C and rinsed in Tris (0.5M, pH 7.5) at room temperature for 5 mins. Sections were then covered in a solution (0.2% Triton X-100, 2% normal serum in PBS) containing a monoclonal antibody directed against digoxigenin (1:50, Roche) and antisera directed against phosphorylated ERK1/2 (1:125) or c-fos (1:4000). A cover slip was placed over the sections and the slides were incubated overnight at 4°C. The cover slips were removed and the sections were washed twice in PBS at room temperature (15min each). Slides were then immersed in a solution containing the appropriate fluorescent-labeled secondary antisera (Alexia 488-labeled rabbit antisera and Cy3-labeled mouse antisera, Molecular Probes, Eugene, OR) for 2hr at room temperature. They were subsequently washed twice in PBS, air dried, coverslipped with Aqua Poly/Mount (Polysciences, Inc, Warrington, PA) and examined under appropriate fluorescent illumination.

Photography—Images were captured on an Olympus BX51 microscope using IPLAB scientific imaging software (Scananalysis Inc., Fairfax, VA). Selected photomicrographs were manipulated using Adobe Photoshop (adjusted for brightness, contrast and size), and each figure was arranged using Adobe Illustrator. For double labeling photomicrographs (Fig. 7), Adobe Photoshop was used to superimpose the images.

Results

Characterization of lesion – Dopamine ablation and 5-HT axonal hyperinnervation

The unilateral 6-OHDA lesion model was purposely employed throughout these experiments, as the intact striatum contra lateral to the lesioned striatum served as an internal control to assess the pre- and post-synaptic striatal alterations. Complete unilateral ablation of the nigro-striatal dopamine innervation was achieved using both intra-nigral (adult lesions) and intra-striatal (neonatal lesions) infusions of 6-hydroxydopamine, and this was verified by the lack of TH-IR axons within the lesioned striatum, compared to robust TH-IR within the intact striatum. The selective destruction of dopamine neurons within the Substantia Nigra (SNr) and Ventral Tegmental Area (VTA), which give rise to striatal dopamine axon terminals, was also verified by the lack of TH-IR neurons in both areas (data not shown). A representative example of unilateral dopamine axonal ablation (absent TH-

IR) is shown in figure 1A (adult lesion) and 1B (neonatal lesion). For all subsequent experiments, only animals showing complete dopamine axonal ablation were analyzed (with a minimum of 6 animals per group).

The serotonergic innervation of the striatum was examined using the plasma membrane serotonin transport protein (SERT) as a marker of 5-HT axons. Figures 1C and 1D represent un-enhanced bright-field montage images of SERT-IR. Following neonatal 6-hydroxydopamine treatment, 5-HT axon terminals hyperinnervate the dopamine depleted striatum (1D), which leads to a 2–3 fold increase in both the numbers of 5-HT axon terminals, and in the amount of 5-HT itself within the lesioned striatum. This does not occur within the intact neonatal striatum, or within the adult lesioned or adult intact striatum (1C). Dark-field images of SERT-IR at a higher magnification are also shown, to further demonstrate the 5-HT axonal hyperinnervation. These images correspond to the boxed regions of Figure 1C and 1D. Figure 1D-1 clearly demonstrates the 5-HT axonal hyperinnervation, which is restricted to the neonatal lesioned striatum.

PCA differentially induces pERK1/2 Map Kinase in striatal neurons of animals lesioned as neonates, but not in animals lesioned as adults

Stimulated 5-HT release within the lesioned striatum has been implicated in multiple forms of plasticity which arise following neonatal 6-hydroxydopamine lesioning (Sivam et al., 1987; Abrous et al., 1992; Brus et al., 1994; El Mansari et al., 1994; Reader and Dewar, 1999; Davids et al., 2002; Moy and Breese, 2002). The second messenger systems responsible for mediating these adaptive changes are unknown; however mounting evidence has shown that the ERK1/2 Map Kinase Pathway plays a central role in neuronal plasticity and adaptation. To address this question, groups of rats were treated with p-Chloroamphetamine (PCA), a potent 5-HT releasing amphetamine derivative, 60 days after neonatal lesioning or 3 weeks after adult lesioning. These measures were adopted so that both groups of animals would be the same age at the time of drug treatment. Animals received 1, 10 and 20mg/kg (free base) and were perfused 15 and 60mins following drug administration. These time points were determined from time course experiments which showed that maximal ERK phosphorylation occurred 15 minutes after PCA, while maximal Fos-induction occurred after 60 minutes. Striatal sections were then processed for immunohistochemistry using antisera directed against phosphorylated ERK1/2 and Fos. Throughout the experiments, Fos induction was used as a marker of drug-induced neuronal activation and to examine the effects, if any, of 6-OHDA lesions on drug-induced neuronal activation (see discussion).

Figure 2 shows that regardless of the dose used, no pERK1/2 staining was seen within the lesioned or intact striatum of animals lesioned as adults (A1–A6). However following 10 or 20mg/kg PCA (B3 and B5), but not 1mg/kg, robust pERK1/2 staining was seen throughout the dorsal striatum of animal lesioned as neonates. From this data a dose of 10mg/kg PCA was chosen and the selectivity of drug-induced ERK1/2 phosphorylation and striatal neuronal activation (Fos induction) was further examined (Fig. 3).

Following 1, 10 or 20mg/kg PCA, Fos-induction was observed in the dorsal striatum of adult lesioned (3A-1) and adult intact (3A-2) striatum (data from 10mg/kg shown). In fact the Fos response to PCA in the adult lesioned striatum (3A-1) was strikingly lower when compared to the intact side (3A-2)(see table 1). With animals lesioned as neonates, robust Fos-IR was also seen throughout the lesioned (3B-1) and intact striatum (3B-2) following 1,10 or 20mg/kg. In these animals comparable numbers of Fos-IR cells were seen on each side (data from 10mg/kg shown). This is consistent with previous studies where Fos induction occurred in the striatum following PCA treatment (Moorman and Leslie, 1996), confirming that striatal neurons had responded to PCA.

PCA administration to animals lesioned as adults resulted in negligible pERK1/2 labeling within the lesioned (3C-1) or intact (3C-2) striatum, regardless of the dose used (data from 10mg/kg shown). Scattered pERK1/2 positive cells were seen in the ventro-medial striatum and nucleus accumbens, but not in the dorsal striatum. However, following neonatal 6-OHDA lesions, robust pERK1/2 staining was seen in a large number of cells throughout the lesioned dorsal striatum (3D-1), following 10 or 20mg/kg PCA (data from 10mg/kg shown here). Again, pERK1/2 positive cells were also found in the nucleus accumbens (3A). Within the intact striatum (3D-2), only scattered pERK1/2 positive cells were seen at higher PCA concentrations in the ventro-medial striatum adjacent to the ventricle, but not within the dorsal striatum. These results show that PCA-induced ERK1/2 phosphorylation is restricted to neurons within the dorsal striatum of neonatal lesioned animals.

DOI also differentially induces pERK1/2 Map Kinase in striatal neurons of animals lesioned as neonates, but not in animals lesioned as adults

Several studies have reported a selective role for 5-HT₂ receptors in the dopamine depleted striatum affecting animal behavior, neuropeptide expression and neurotransmitter receptor expression (Gresch and Walker, 1999; Basura and Walker, 2001; Campbell et al., 2001; Campbell and Walker, 2002; Bishop et al., 2003; Bishop et al., 2004). Indeed the level of the 5-HT_{2A} receptor expression increased selectively in direct pathway striatal neurons following neonatal dopamine depletion (Numan et al., 1995; Laprade et al., 1996). Having demonstrated that PCA-stimulated 5-HT release induces ERK1/2 phosphorylation in neonatal lesioned striatum, we further examined the role of striatal 5-HT₂ receptors specifically, by administering the select 5-HT₂ receptor agonist DOI to groups of rats that were either lesioned as neonates or as adults. 1, 5 or 10mg/kg DOI was administered 60 days (neonates) or 3 weeks (adult) after 6-OHDA lesions, and animals were perfused 15mins (pERK1/2) and 60mins (Fos), in accordance with the previous experiments. Once again Fos induction was used as an indicator of drug-induced neuronal stimulation.

Figure 4 shows that regardless of the dose used, no pERK1/2 staining was seen within the lesioned or intact striatum of animals lesioned as adults (A1–A6). However following 1, 5 or 10mg/kg DOI (B1, B3 and B5), robust pERK1/2 staining was seen throughout the dorsal striatum of animal lesioned as neonates. From this data a dose of 5mg/kg PCA was chosen and the selectivity of drug-induced ERK1/2 phosphorylation and striatal neuronal activation (Fos induction) was further examined (Fig. 5).

60mins following DOI (1, 5 or 10mg/kg), robust Fos-IR was seen throughout the adult lesioned (5A-1) and intact striatum (5A-2) (data from 5mg/kg shown). This is consistent with previous studies where Fos induction was reported in the striatum following DOI treatment (Rouillard et al, 1996), confirming that DOI stimulates striatal neurons. DOI administration to neonatal lesioned rats also gave rise to robust Fos-IR throughout the lesioned (5B-1) and intact (5B-2) striatum (data from 5mg/kg shown). The overall Fos response to DOI was less intense compared to that seen after PCA (see table 1).

Adult rats lesioned with 6-OHDA and treated with DOI showed no detectable pERK1/2 within the lesioned (5C-1) or intact (5C-2) striatum, regardless of the dose used (data from 5mg/kg shown). In contrast, 15mins after the administration of 1, 5 or 10mg/kg DOI, robust pERK1/2 was seen throughout the lesioned striatum (5D-1) in a distribution similar to that following PCA, albeit to a slightly lesser degree. Labeled neurons were also seen within the nucleus accumbens. Virtually no pERK1/2 was detected in the intact (5D-1) striatum of neonatal lesioned animals (5D-1), even at the higher drug doses. These results show that ERK1/2 phosphorylation is mediated via 5-HT₂ receptors, but only within the neonatal lesioned striatum.

Effects of 6-OHDA lesions on PCA and DOI induced Fos-induction in the dorsal striatum

Data from Table 1 shows that adult 6-OHDA lesions severely affect the response of striatal neurons to PCA. As dopamine nerve terminals have been destroyed, the Fos-IR most likely reflects the response of striatal neurons 5-HT, released from presynaptic terminals by PCA. When animals were lesioned with 6-OHDA as neonates, there was a greater Fos-IR response in the lesioned striatum compared to the intact striatum following PCA. In this case the 5-HT hyperinnervation of the neonatal lesioned striatum provided significantly more 5-HT, which allowed a more robust “striatal” response to PCA (see discussion).

Ketanserin blocks DOI induced ERK1/2 phosphorylation and Fos expression

To further characterize the role of 5-HT₂ receptors neonatal lesioned animals were treated with a select 5-HT₂ receptor antagonist Ketanserin (Harvey, 1996, 2003; Barnes and Sharpe, 1999) (1–40mg/kg), 30mins prior to DOI administration (1–5mg/kg). Figure 6A and 6C shows that pretreatment with vehicle and subsequent DOI treatment (5mg/kg) gave the robust Fos response in the lesioned (6A-1) and unlesioned (6A-2) striatum, together with robust pERK1/2 staining in the neonatal lesioned striatum (6C-1), as previously described. However, Ketanserin (data from 40mg/kg shown here) completely blocked DOI-mediated Fos induction in the lesioned (6B-1) and intact (6B-2) striatum, together with ERK 1/2 phosphorylation in the lesioned striatum (6D-1). Doses of Ketanserin between 10–40 mg/kg showed an increasing reduction in both Fos and pERK. This indicated that Ketanserin antagonized 5-HT₂ receptors in a dose-dependant manner, and the effects seen were not related to the high dose of antagonist used to block the effects of 5mg/kg DOI. In fact, the effects of 1mg/kg DOI were completely blocked by 5–10mg/kg Ketanserin, again indicating a dose-dependant effect (data not shown). These data further demonstrate that neonatal dopamine depletion results in a novel coupling of 5-HT₂ receptors to the ERK1/2 MAP Kinase pathway within the neonatal lesioned striatum.

Localization of pERK1/2 to direct pathway neurons following either PCA or DOI

Functional dichotomy within the striatum is expressed through the segregation of the medium spiny neurons into a direct and indirect pathway (Gerfen et al., 1990). The medium spiny neurons represent approximately 95% of the total number of striatal neurons, dividing equally between the direct and indirect systems. Direct pathway neurons express dynorphin, substance P and D1 dopamine receptors, while indirect pathway neurons express enkephalin and D2 dopamine receptors. To further examine the role of 5-HT within the neonatal lesioned striatum, double labeling of striatal neurons following DOI treatment was performed to address two important questions: (1) What neuronal phenotypes in the striatum responded to DOI treatment and (2) in which striatal neurons is drug-induced pERK1/2 found.

Double labeling of sections for Fos-IR (immunohistochemistry) and with a digoxigenin-labeled riboprobe directed against enkephalin mRNA (in situ hybridization) showed that Fos induction occurred within both enkephalin positive and enkephalin negative striatal neurons (7F), confirming that both the direct and indirect striatal systems were activated following DOI treatment. Combined fluorescent double labeling for pERK1/2 and again enkephalin mRNA (7C), clearly demonstrates that all of the cells which showed pERK1/2 staining following DOI treatment did not express enkephalin, and therefore drug induced pERK1/2 is located exclusively within direct pathway neurons of the dorsal striatum. The double labeling data also shows that ERK1/2 phosphorylation is not simply due to a select pharmacological effect of DOI on direct pathway neurons (as Fos was seen in both direct and indirect neurons after DOI), but it results from neonatal lesioned-induced coupling of 5-HT₂ receptors to the ERK1/2 Map Kinase pathway.

Discussion

Here we have shown that neonatal 6-OHDA lesions result in a novel coupling of 5-HT₂ receptors to the ERK1/2 MAP Kinase cascade, a signaling pathway which plays a central role in neuronal plasticity. Normal signaling through 5-HT₂ receptors involves coupling (positively) of all three 5-HT₂ receptor subtypes to phospholipase C (PLC), which does not involve activation of the ERK1/2 Map Kinase pathway. However, following neonatal but not adult lesions, stimulated 5-HT release (PCA) or activation of 5-HT₂ receptors with a select agonist (DOI) results in robust ERK1/2 phosphorylation throughout the neonatal lesioned striatum. This drug induced ERK1/2 phosphorylation was blocked by pre-treating animals with the select 5-HT₂ antagonist Ketanserin. Double labeling experiments showed that drug-induced ERK1/2 phosphorylation was found exclusively within striatal direct pathway neurons, even though both direct and indirect pathway neurons had responded to drug treatment. The adaptations in 5-HT neurotransmission within the neonatal lesioned striatum are likely to have profound effects on the function of the direct pathway and ultimately motor behavior.

5-HT₂ receptors couple to ERK1/2 following neonatal 6-OHDA

Mounting evidence has strongly implicated 5-HT in neuronal plasticity following neonatal dopamine depletion, and in particular 5-HT interacting with striatal 5-HT₂ receptors (Sivam et al., 1987; Abrous et al., 1992; Brus et al., 1994; El Mansari et al., 1994; Reader and Dewar, 1999; Davids et al., 2002; Moy and Breese, 2002). However the intracellular mechanisms responsible for these adaptations are poorly understood. 5-HT₂ receptors are expressed on both direct and indirect pathway neurons (Ward and Dorsa, 1996), and they are coupled to the PLC signaling cascade. Here we show that neonatal 6-OHDA lesions result in a novel coupling of 5-HT₂ receptors to ERK1/2 MAP Kinase Pathway, as demonstrated by robust ERK1/2 phosphorylation following PCA or DOI. Data from double labeling experiments showed that this occurs selectively within direct pathway neurons of the dorsal striatum. Ketanserin blocked the drug induced ERK1/2 phosphorylation, which further demonstrates the specific coupling between 5-HT₂ receptors and the ERK1/2 Map Kinase pathway. The question arises as to why these drugs do not activate ERK1/2 Map Kinase within indirect pathway neurons, as they also express 5-HT₂ receptors. In order to control for the effects of both drugs on striatal neurons following both adult and neonatal lesions, we used Fos-IR as a general indicator of receptor mediated neuronal stimulation. Previous studies have shown that both PCA and DOI treatments result in robust Fos induction in striatal neurons (Moorman and Leslie, 1996; Rouillard et al, 1996), and we have confirmed those findings here. Furthermore, data from our double labeling experiments clearly demonstrates Fos-IR in both enkephalin positive and enkephalin negative neurons, demonstrating that both the direct and indirect pathways had responded to PCA and DOI. This result also shows that ERK1/2 phosphorylation in direct pathway neurons is not attributed to selective stimulation of these neurons by PCA or DOI (Fos data showed that both direct and indirect striatal neurons were activated following drug treatment). Therefore the adaptation within direct pathway neurons following neonatal dopamine depletion involves a novel coupling of 5-HT₂ receptors to the ERK1/2 Map Kinase pathway.

Pre and post-synaptic alteration in striatal serotonergic system following neonatal dopamine depletion

Following neonatal destruction of the dopaminergic fibers, two important alterations occur within the serotonergic system; hyperinnervation by striatal targeted 5-HT axon terminals (Stachowiak et al., 1984; Blue and Molliver, 1987; Towle et al., 1989), and increased 5-HT_{2A} receptor expression on direct pathway neurons (Laprade et al., 1996; Basura and Walker, 1999). Therefore both the pre and post-synaptic serotonin systems have been

altered. Activation of the dorsal raphe gives rise to a 2–3 fold increase in striatal 5-HT release from hyperinnervated terminals (Molina-Holgado et al., 1994), compared to unlesioned controls. Taken together with our current findings, the increased 5-HT release and increased 5-HT_{2A} receptor expression is likely to have profound effects on direct pathway striatal neurons and indeed on the output of the direct pathway itself.

Although pERK1/2 was found throughout the striatum following PCA or DOI treatment, the highest levels were seen within the rostral aspect of the lesioned striatum.

The striatum receives 5-HT axons from the Raphe nucleus (Lidov and Molliver, 1982), where dorsal raphe neurons project to the striatum and nucleus accumbens while the median raphe neurons project selectively to the nucleus accumbens (Brown and Molliver, 2000). Under normal developmental conditions, the caudal regions of the striatum are more densely innervated by 5-HT axon terminals than the rostral areas (Ternaux et al., 1977). However, following neonatal 6-OHDA lesions the rostral regions of the striatum become hyperinnervated with 5-HT axon terminals, an effect attributed to increased terminal sprouting following dopamine axonal destruction (Kostrzewa et al., 1998). Thus, there is a direct anatomical correlation between the hyperinnervated 5-HT axon terminals and striatal regions where maximal drug-induced activation of the ERK1/2 MAP Kinase pathway was seen.

Analysis of Fos-IR following PCA or DOI treatment also provided useful information concerning the effects of 6-OHDA lesioning on the response of striatal neurons, most notably with respect to PCA-induced neurotransmitter release. The Fos response to PCA in the adult lesioned striatum was strikingly lower when compared to the intact side (see supplemental data). As all amphetamines can release dopamine to varying degrees, which itself can induce robust Fos-IR in striatal neurons (Graybiel et al., 1990; Bhat et al., 1992), our data suggests that on the intact side both 5-HT and dopamine stimulated release are responsible for Fos expression, while the lack of dopamine on the lesioned side leaves only 5-HT to induce the Fos response. With animals lesioned as neonates, robust Fos-IR was seen throughout both the lesioned and intact striatum following PCA treatment, with comparable numbers of Fos-IR cells on each side. Our data suggests that the 5-HT hyperinnervation seen in neonatal lesioned animals may compensate for the dopamine deficit, allowing a more robust drug-induced stimulation of striatal neurons (see Table 1).

Taking into account our current data, it is important to re-evaluate previous reports showing that activation of 5-HT₂ receptors can restore the level of striatal neuropeptide expression within the lesioned striatum. Specifically, decreased PPT in direct pathway neurons was returned to control levels following PCA treatment (Gresch and Walker, 1999; Basura and Walker, 2000) or by the 5-HT₂ receptor agonist DOI (Campbell et al., 2001; Campbell and Walker, 2002). Our current data shows that both drugs selectively phosphorylate ERK1/2 within direct pathway neurons, implying that stimulation of this pathway may underlie the restoration of PPT expression. However, earlier studies demonstrated that DOI treatment restored PPT expression to control levels in animals lesioned both as adults and as neonates, and our own data clearly shows that following adult lesions, DOI treatment does not lead to ERK1/2 phosphorylation in striatal neurons. Nevertheless, as ERK1/2 Map Kinase is a central mediator of neuronal plasticity, it would be interesting to see if MEK inhibitors (which block MAPK) affect PCA or DOI restoration of gene expression following neonatal 6-OHDA lesions.

Furthermore, the role of 5-HT mediated ERK1/2 phosphorylation in regulating motor systems remains to be determined. Drugs previously used to examine the role of striatal 5-HT₂ receptors in motor function after neonatal lesions are shown here to selectively

phosphorylate ERK1/2 within direct pathway neurons. A recent report which showed that MEK inhibition actually potentiated hyperactivity in neonatal lesioned animals would imply a functional role for this signaling cascade (Tessmer et al., 2003). Activation of ERK1/2 MAP Kinase in striatal direct pathway neurons may help explain the paradoxical therapeutic effect of amphetamine on hyperactivity.

Alterations and adaptations within second messenger signaling cascades

Lesioning adult rats with 6-OHDA results in a novel coupling of D1-dopamine receptors to the ERK1/2 Map kinase pathway in the dorsal striatum, and this is responsible for the supersensitive response of direct pathway neurons to D1 agonist treatments (Gerfen et al., 2002). The supersensitive response in the dopamine-depleted dorsal striatum differs markedly from the normal coupling of D1 receptors through PKA signaling mechanisms (Konradi et al, 1994) in terms of the sensitivity to D1 agonist treatments, regulation of neuropeptide expression, desensitization to repeated treatments, and dependence on NMDA co-activation (Steiner and Gerfen, 1993, 1995; Keefe and Gerfen, 1998). This novel lesion-induced coupling of the D1-dopamine receptor to an additional signal-transduction system significantly alters the function of direct-pathway neurons. The present study shows that following neonatal lesions of the nigrostriatal dopamine system, there is a novel lesion-induced coupling of 5HT₂ receptors to the ERK1/2 Map Kinase pathway, which also occurs selectively within direct pathway neurons of the dorsal striatum. Our data suggests that 5-HT hyperinnervation, increased 5-HT_{2A} receptors expression, and lesioned-induced coupling of 5-HT₂ receptors to ERK1/2 Map Kinase, are likely to have a profound effect on the function of direct pathway neurons.

Even though 5-HT and 5-HT₂ receptors play a central adaptive role in direct pathway neurons following neonatal 6-OHDA lesions, mounting evidence suggests that D1 dopamine receptors are co-activated with 5-HT_{2A} receptors following drug treatment. This synergistic effect leads to a more striking up-regulation of gene expression in direct pathway neurons when compared to 5-HT receptor stimulation alone (Campbell et al., 2001). Our previous work demonstrated that following adult 6-OHDA lesions, D1 dopamine receptors exhibit a novel coupling to the ERK1/2 Map kinase pathway (Gerfen et al., 2002). In addition, D1 agonist administration to animals lesioned with 6-OHDA as neonates leads to robust ERK1/2 phosphorylation in the striatum (Papadeas et al., 2004). We have also seen this effect, specifically D1 dopamine receptor mediated ERK1/2 phosphorylation in direct pathway neurons in our unilateral neonatal 6-OHDA model (unpublished observation). Therefore following neonatal 6-OHDA lesions, both 5-HT₂ and D1 dopamine receptors can activate the ERK1/2 MAP Kinase pathway, which they do not use under normal conditions. Our data further extends the behavioral and molecular studies and implies a functional interaction involving 5-HT and dopamine receptors within direct pathway neurons, which they mediate in part via phosphorylation of ERK1/2. The molecular, cellular and behavioral consequences of this synergistic mechanism remain to be determined.

It is important to reiterate the significance of lesioned-induced coupling of 5-HT₂ receptors to ERK1/2 Map Kinase. The classical view of receptor mediated signaling involves individual receptors coupling to a single second messenger system. Indeed the classification of 5-HT receptors within a receptor family is based on sequence homology and coupling to specific second messenger cascades (Molineaux et al., 1989; Julius et al., 1990, 1991). However growing evidence suggests that G-protein coupled receptors (GPCR) are able to couple to multiple second messenger systems, either directly or indirectly (Lin et al., 2002; Castro et al., 2003; Cordeaux et al., 2004; Clarke et al., 2005). Our data shows that following significant alterations within the external environment, GPCR's on direct pathway neurons can couple to alternative second messenger cascades. This further implies that receptor coupling to second messenger systems is itself "plastic", and that receptors are not

“hard-wired” to just one second messenger system under certain conditions. These findings may help to elucidate the intracellular mechanisms by which neurons respond to major adaptations in the extracellular neurochemical environment.

Conclusion

Plasticity within differentiated neurons is essential for successful adaptation within the ever changing cellular environment. Increasing evidence suggests that this is achieved through activation of the ERK1/2/MAP Kinase pathway. This signaling cascade plays a central role in cellular learning and memory (Sweatt, 2001; Thomas and Huganir, 2004), which it mediates in part through its actions on LTP and LTD. The downstream targets of this signaling cascade include multiple IEG's, and these transcription factors are themselves central regulators of gene expression (Treisman, 1996; West et al., 2002). Activation of the same transcription factors has been reported following stimulation at dopamine or serotonin receptors; however this does not involve ERK1/2/MAP Kinase and is mediated through the actions of cAMP, PKA and PLC respectively. Lesion-induced coupling of the ERK1/2/MAP Kinase cascade to 5-HT₂ receptors represents adaptation of direct pathway neurons to dopamine depletion. As ERK1/2/MAP Kinase plays a central role in both cellular and synaptic plasticity, 5-HT mediated activation of this pathway could provide a compensatory mechanism allowing direct pathway neurons to survive and remain functional within the post-lesioned striatum.

Acknowledgments

This research was supported by the Intramural Research Program of the NIMH, NIH.

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Figure 1. Unilateral 6-OHDA lesioning of adult and neonatal rat pups leads to permanent changes in both the dopaminergic and serotonergic systems within the striatum

Bright-field images show that unilateral 6-OHDA lesions lead to complete destruction of dopamine axon terminals (1A, 1B), as demonstrated by the lack of tyrosine hydroxylase immunoreactivity (TH-IR) within the lesioned striatum. The dopamine terminal field contralateral to the lesioned striatum remains intact.

Figures 1C and 1D show bright field montage images of SERT-IR staining in sections adjacent to those stained for TH-IR. Following a lesion, 5-HT axonal hyperinnervation occurs, but only within the neonatal lesioned striatum (1D).

Figures 1C-1, 1C-2 and 1D-1, 1D-2 show representative high magnification dark-field images of serotonin axons within the striatum (SERT-IR), following unilateral adult and neonatal 6-hydroxydopamine lesions. Images were captured within striatal regions defined by the rectangular boxes in figure 1C and 1D. Following adult lesions, SERT-IR within the lesioned (1C-1) and intact (1C-2) striatum was unaltered. However, neonatal dopamine ablation resulted in 5-HT axonal hyperinnervation of the lesioned striatum (1D-1), but not the intact striatum (1D-2). Scale bar = 1mm for 1A, 1B; 120 μ m for 1C, 1D; 50 μ m for 1C-1, 1C-2, 1D-1 and 1D-2.

Figure 2. PCA dose response: stimulated 5-HT release induces ERK1/2 phosphorylation in striatal neurons following neonatal but not adult 6-OHDA lesions

Low magnification photographs show representative examples of PCA-induced pERK1/2 staining throughout the striatum following either adult (2A) or neonatal (2B) 6-OHDA lesions. Images shown represent animals treated with 10mg/kg PCA. The boxed area within the dorsal striatum corresponds to the higher magnification micrographs shown at several different PCA doses: 1, 10 and 20mg/kg. All animals were perfused 15 minutes after drug treatment.

Drug administration to animals lesioned as adults failed to induce robust ERK1/2 phosphorylation within the dorsal striatum of lesioned (A1, A3 and A5) or intact (A2, A4 and A6) animals, regardless of the dose used. Scattered pERK1/2 staining was seen in the ventral striatum, particularly within the shell region of the nucleus accumbens.

Treatment with either 10 or 20mg/kg PCA, but not 1mg/kg, gave rise to robust ERK1/2 phosphorylation throughout the dorsal striatum of animals lesioned as neonates (B3 and B5). Scattered pERK1/2 staining was seen within the intact striatum at the higher toxic drug doses (B4 and B6), but mainly within ventral regions of the striatum. In both lesioned and intact animals, pERK1/2 staining was seen throughout the nucleus accumbens, particularly within the shell region.

This data shows that increasing doses of PCA stimulate ERK1/2 phosphorylation, which is restricted to the dorsal striatum of animals lesioned as neonates. Scale bar = 1mm in 2A and 2B; 100µm in all other photomicrographs.

Figure 3. PCA differentially induces ERK1/2 phosphorylation in striatal neurons, following neonatal 6-OHDA lesions

PCA (10mg/kg) administration induced robust Fos-IR throughout the adult lesioned (3A-1) and intact (3A-2) striatum as well as in the neonatal lesioned (3B-1) and intact (3B-2) striatum, demonstrating that PCA activates striatal neurons. Drug administration to animals lesioned as adults failed to induce ERK1/2 phosphorylation within lesioned (3C-1) or intact (3C-2) striatum, regardless of the dose used. Staining within the nucleus accumbens was still observed. In contrast, treatment with 10mg/kg PCA produced robust ERK1/2 phosphorylation throughout the dorsal striatum of animals lesioned as neonates (3D-1). Scattered pERK1/2 staining was seen within the intact (3D-2) striatum at higher drug doses. In both neonatal lesioned and intact animals, pERK1/2 staining was seen throughout the

nucleus accumbens, particularly within the shell region. Scale bar = 1 mm in 3A, 3B, 3C and 3D; 100 μ m in all other photomicrographs.

Figure 4. DOI dose response: a select 5-HT₂ receptor agonist induces ERK1/2 phosphorylation in striatal neurons following neonatal but not adult 6-OHDA lesions

Low magnification photographs show representative examples of DOI-induced pERK1/2 staining throughout the striatum following either adult (4A) or neonatal (4B) 6-OHDA lesions. Images shown represent animals treated with 5mg/kg DOI. The boxed area within the dorsal striatum corresponds to the higher magnification micrographs shown at several different DOI doses: 1, 5 and 10mg/kg. All animals were perfused 15 minutes after drug treatment.

Drug administration to animals lesioned as adults failed to induce robust ERK1/2 phosphorylation within the dorsal striatum of lesioned (A1, A3 and A5) or intact (A2, A4 and A6) animals, regardless of the dose used. Scattered pERK1/2 staining was seen in the the nucleus accumbens.

Treatment with 1, 5 or 10mg/kg PCA gave rise to robust ERK1/2 phosphorylation throughout the dorsal striatum of animals lesioned as neonates (B1, B3 and B5). Virtually no pERK1/2 staining was seen within the intact striatum regardless of the dose (B2, B4 and B6). In both lesioned and intact animals, scattered pERK1/2 staining was seen in the nucleus accumbens, particularly within the shell region.

This data shows that select activation of 5-HT₂ receptors leads to robust ERK1/2 phosphorylation exclusively in the dorsal striatum of animals lesioned as neonates. Scale bar = 1mm in 4A and 4B; 100µm in all other photomicrographs.

Figure 5. 5-HT₂ receptor agonist treatment differentially induces ERK1/2 phosphorylation in striatal neurons, following neonatal 6-OHDA lesions

DOI (5mg/kg) administration produced robust Fos-IR throughout the adult lesioned (5A-1) and intact (5A-2) striatum as well as in the neonatal lesioned (5B-1) and intact (5B-2) striatum, demonstrating that DOI activates striatal neurons. Drug administration to animals lesioned as adults failed to induce ERK1/2 phosphorylation within lesioned (5C-1) or intact (5C-2) striatum, regardless of the dose used. In contrast, robust ERK1/2 phosphorylation was seen throughout the striatum of animals lesioned as neonates (5D-1) (data from 5mg/kg shown here) in a distribution similar to that seen following PCA treatment. Remarkably, little pERK1/2 staining was seen within the intact striatum (5D-2), regardless of the dose used. Scale bar = 1mm in 5A, 5B, 5C and 5D; 100 μ m in all other photomicrographs.

Figure 6. Ketanserin, a select 5-HT₂ receptor antagonist, completely abolishes DOI-induced ERK1/2 phosphorylation in direct pathway neurons of animals lesioned as neonates

Animals pretreated with vehicle and then with DOI (5mg/kg) showed the characteristic Fos response in both the lesioned (6A-1) and intact (6A-2) striatum, as well as the characteristic pERK1/2 response (6C-1) in the neonatal lesioned striatum. Pretreatment of neonatal lesioned rats with Ketanserin (40mg/kg), completely blocked DOI (5mg/kg) induced Fos-IR (6B-1, 6B-2) and ERK1/2 phosphorylation (6D-1). Therefore ERK1/2 phosphorylation in striatal neurons is mediated via drug induced activation of 5-HT₂ receptors. Scale bar = 1mm in 6A, 6B, 6C and 6D; 100µm in all other photomicrographs

Figure 7. Drug induced ERK1/2 phosphorylation is restricted to striatal neurons of the direct pathway

Double labeling was performed using a digoxigenin-labeled ribonucleic probe complementary to enkephalin mRNA sequence (*in situ* hybridization) and antisera directed against pERK1/2 or Fos (immunohistochemistry). Both probes were detected with fluorescent secondary antibodies. pERK1/2 positive neurons (7A, arrow head) and enkephalin containing neurons (7B, arrow) showed no overlap when images were superimposed (7C). As enkephalin is selectively expressed by striatal neurons of the indirect pathway, PCA (10mg/kg) or DOI (5mg/kg) induced phosphorylation of ERK1/2 is restricted to direct pathway neurons. The sample images shown are from neonatal lesioned rats treated with DOI. The same result was obtained from neonatal animals treated with PCA (data not shown). Drug induced Fos-IR was found in both direct and indirect pathway neurons, in both the lesioned and intact striatum (7D-F: white arrow head = Fos⁺/Enk⁻ direct pathway neuron; blue arrow head = Fos⁺/Enk⁺ indirect pathway neuron; white arrow = Enk⁺/Fos⁻ indirect pathway neuron). These data show that DOI activates both direct and indirect pathways neurons, demonstrated by Fos induction, but ERK1/2 phosphorylation is restricted to direct pathway neurons. Scale bar = 16μm.

Figure 8. Adaptation of second messenger signaling in direct pathway neurons following neonatal dopamine depletion

This diagram summarizes the switch from dopamine and serotonin mediated transmission (top), to serotonin mediated transmission (bottom) in direct pathway striatal neurons following neonatal 6-OHDA lesions. Within the intact striatum (top), dopamine acting at D1 dopamine receptors, together with serotonin acting at 5-HT₄ and 5-HT₆ receptors, results in DARPP-32 phosphorylation via cAMP and PKA. Activation of 5-HT_{2A} receptors also leads to DARPP-32 phosphorylation, this time through PLC and casein kinase 1. Although DARPP-32 is not the only target of PKA and PLC, it represents a central regulatory mechanism within striatal projecting neurons, mediating the effects of both dopamine and serotonin (Svenningsson et al., 2002).

Following neonatal 6-OHDA lesions (bottom), signaling via dopamine D₁ receptors is redundant, as dopamine is absent. Under these conditions, DARPP-32 phosphorylation is under the control of the 5-HT receptors. The post-lesion striatum is characterized by increased 5-HT_{2A} receptor expression and 5-HT axonal hyperinnervation. Both of these alterations are likely to significantly enhance the ability of 5-HT to regulate DARPP-32 phosphorylation and therefore neuronal function. In addition, 5-HT₂ receptors couple to the ERK1/2/MAP Kinase pathway, which is crucial for neuronal and synaptic plasticity. This pathway is not used under normal conditions (see top). Activation of ERK1/2 could provide an alternative signaling route to the nucleus, leading to the activation of transcription factors that regulate neuronal gene expression, and ultimately neuronal plasticity. ERK1/2 activation could represent a mechanism which allows direct pathway neurons to adapt and remain functional in the absence of dopamine.

Table 1
Effects of 6-OHDA lesions on PCA and DOI induced Fos-induction in the dorsal striatum

The number of cells listed for each animal represents the average number of Fos-IR striatal cells within a 250µm square area, with 5 samples taken from each striatal region. PCA, 10mg/kg p-Chloroamphetamine; DOI, 5mg/kg DOI

	Adult		Neonate	
	Lesion	Intact	Lesion	Intact
PCA	17.13 ± 1.77	40.3 ± 2.5	58.3 ± 2.3	42.9 ± 1.1
DOI	18.3 ± 1.9	31.4 ± 1.3	43.7 ± 9.2	35.18 ± 4.7