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ERK1, 2, and 5 expression and activation in dopaminergic brain regions during postnatal development

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ABSTRACT

Degeneration and dysfunctioning of dopaminergic neurons in the midbrain have been associated with serious neurodegenerative and neuropsychiatric disorders. Elucidating the underlying neurobiology of these neurons during early postnatal development may provide important information regarding the etiology of these disorders. Cellular signaling pathways have been shown to regulate postnatal neuronal development. Among several signaling pathways, extracellular-regulated mitogen kinases (ERK) 1, 2, and 5 have been shown to be crucial for the survival and function of dopaminergic neurons. In this study, the basal expression and activation of ERK1, 2, and 5 were studied during postnatal development in regions rich in DA cells and terminals. In the striatum (STR) and ventral mesencephalon regions of the substantia nigra (SN) and ventral tegmental area (VTA), ERK5 expression and activation were high during early postnatal days and declined with aging. Interestingly, sharp increases in phosphorylated or activated ERK1 and ERK2 were observed at postnatal day (PND) 7 in the SN and VTA. In contrast, in the STR, the levels of phosphorylated ERK1 and 2 were significantly higher at PND0 than at any other PND examined. Overall, the understanding of alterations in ERK signaling in regions rich in DA cells and DA terminals during postnatal neuronal development may provide information about their role in regulation of dopamine neuronal development which may ultimately provide insight into the underlying mechanisms of dopamine neurodegeneration.

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1. Introduction

Dopamine (DA)-containing cells are present in the substantia nigra (SN) and ventral tegmental area (VTA) and project to several key telencephalic regions, including the neostriatum, nucleus accumbens, and frontal cortex. These neurons have many critical roles and impairments in their survival and signaling contribute to many clinical disorders, including Parkinson's disease, depression, and schizophrenia (Dailly et al., 2004; Carlsson et al., 2001; Sesack and Carr, 2002).

Early prenatal and postnatal development of dopaminergic midbrain neurons, their projections, and their function are precisely controlled by complex network of transcription factors, such as Nurr1, Lmx1b, Pitx3, and En1/En2 (Riddle and Pollock, 2003; Ries et al., 2009). Whereas considerable progress has been made in our understanding of the prenatal and postnatal development of DA neurons, including neuron number, axon guidance, target contact, natural cell death events, and DA homeostasis (Janec and Burke 1993; Oo and Burke 1997; Smidt et al., 2003; Rose et al., 2014), cell signaling pathways within DA cells and regions rich in DA terminals that may alter and/or control these developmental processes are not as well studied. In a study by Ries et al. (2009), the postnatal development of DA neurons of the substantia nigra in vivo was shown to be regulated, at least in part, by the signaling protein Akt/protein kinase B. Similarly, the extracellular signal-regulated kinase (ERK) pathways have been demonstrated to be important for dopaminergic neuronal development, survival, and function (Cavanaugh et al., 2006a,b; Kim et al., 2006; Yoon et al., 2011; Parmar et al., 2014). For example, using primary dopaminergic mesencephalic cultures, we have shown that pharmacological inhibition of ERK5, and to a lesser extent ERK1 and 2, resulted in a significant loss of viability of dopaminergic neurons at day in vitro (DIV) 2, 4, 6, and 8 (Parmar et al., 2014). Moreover, others have shown that the regulation of dopaminergic neuronal development is regulated by D2 receptor-mediated ERK activation (Kim et al., 2006; Yoon et al., 2011; Yoon and Baik, 2013). These data suggest that ERK pathways are necessary for basal survival of dopaminergic

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neurons during early time points and may play crucial role in the development of dopaminergic brain regions.

Previous studies have shown that ERK5 levels are highest during early prenatal development of the brain (Liu et al., 2003, 2006). In contrast to ERK5, low expression of ERK1 and ERK2 have been observed during early development of the nervous system with increases in the expression of ERK1 and 2 noted during later postnatal development (Boulton et al., 1991; Liu et al., 2003). However, postnatal changes in ERK1, 2, and 5 expression and activation in specific DA-rich brain regions, have not been previously examined. Understanding the alterations that occur in ERK expression and activation in these regions during development may provide insight into intracellular signaling mechanisms that underlie dopaminergic neuronal survival, target selection, and function. Further, it could help to understand the intracellular signaling changes that may lead to the age-related loss of dopaminergic neurons and terminals in these regions. Hence, the current study is focused on examining the expression and activation of these signaling proteins during early postnatal development in the substantia nigra, VTA, and striatum.

2. Materials and methods

2.1. Animals

Time pregnant and adult Sprague-Dawley rats (Charles River) were adapted to a 12:12 h light: dark cycle and provided with water and Purina Rat Chow ad libitum. All procedures were conducted in accordance with the guidelines for the NIH Care and Use of Laboratory Animals and approved by the University of Pittsburgh Institutional Animal Care and Use Committee (IACUC). Rats from different post developmental stages (postnatal days 0, 7, 10, 14 and 2–3 mo; $n = 3$) and adult rats (2–3 mo) were used.

2.2. Materials and tissue preparation

The SN, VTA, and striatal (dorsal + ventral) tissues were isolated from pup brain at day 0, 7, 10, 14, and from 2 to 3 month, flash frozen on dry ice, and stored at −80 °C. All reagents were purchased from Sigma–Aldrich (St. Louis, MO) unless otherwise specified. Tissue samples were sonicated in ice-cold 1:20 (weight/volume) lysis buffer containing 20 mM Tris, pH 6.8, 137 mM NaCl, 25 mM β-glycerophosphate, pH 7.14, 2 mM NaPPi, 2 mM EDTA, 1 mM Na_3VO_4, 1% Triton X-100, 10% glycerol, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 2 mM benzamidine, 0.5 mM DTT and 1 mM PMSF) using a 2% pulse. Total homogenate was centrifuged at 11,000 rpm for 30 min at 4 °C and the supernatant was collected and stored at -80 °C until analysis.

2.3. Western blot analysis

Total protein content was assessed by Bradford Bio-Rad protein assay (Bio-Rad, Hercules, CA) and 60 μg of protein was loaded on an 8% SDS-PAGE gel and transferred to a nitrocellulose membrane (Licor Biosciences, Lincoln, NE). After transfer, membranes were washed for 5 min with 1X PBS and blocked for 1 h in a casein blocking buffer (Licor Biosciences) at room temperature. Membranes were then incubated overnight at 4 °C in primary antibody in casein blocking buffer with 0.1% Tween-20. Antibodies included rabbit anti-phospho-ERK1/2 (dilution – 1:1000, Cat. No. 9101, Cell Signaling, Beverly, MA), mouse anti-total ERK1/2 (dilution – 1:2000, Cat. No. 9107, Cell Signaling), and rabbit anti-total ERK5 (dilution – 1:1,000, Cat. No. 3372, Cell Signaling). Mouse anti-α-Tubulin (dilution – 1:10,000, Cat. No. T5168, Sigma–Aldrich) was used as a loading control. After incubation with primary antibody, blots were washed in 1X PBS solution with 0.1% Tween-20 (1X PBS-T) and incubated with goat anti-rabbit (dilution –1:20,000, Cat. No. 926-68021, Licor Biosciences) and goat anti-mouse (dilution – 1:20,000, Cat. No. 926-32210, Licor Biosciences) secondary antibodies for 1 h at room temperature. After washing the membranes with 1X PBS-T, the protein bands were visualized on an Odyssey Infrared Imager and quantified with Odyssey software (Licor Biosciences).

2.4. Statistical analysis

GraphPad Prism 5 Software (San Diego, CA) was used for statistical analysis. Data are expressed as mean ± SEM. Statistical comparison was performed using a one-way analysis of variance (ANOVA) followed by post hoc Dunnet's test. Statistical significance was defined at $p < 0.05$.

3. Results

3.1. ERK5, ERK1, and ERK2 levels in the ventral mesencephalon during postnatal brain development.

ERK5 and phospho-ERK5 (p-ERK5) expression levels decreased during postnatal development in the SN with a significant decline in ERK5 expression and in p-ERK5 starting at PND10 as compared to PND0 (Fig. 1). ERK5 activation (*i.e.,* p-ERK5/ERK5) decreased significantly starting at PND14 as compared to PND0 (Fig. 1). Significant increases in p-ERK1 and p-ERK2 expression were observed at PND7, followed by declines at PND10 and PND14 to the levels noted at PND0 and adult age (Fig. 1). Similar to p-ERK1 and p-ERK2 expression, ERK1 and ERK2 activation (*i.e.* p-ERK1/ERK1 and p-ERK2/ERK2) increased significantly at PND7, followed by decline at PND10 and PND14 (Fig. 1). Similar results were observed in the VTA with respect to ERK5 and p-ERK5 expression changes (Fig. 2). However, biphasic increases in p-ERK1 and p-ERK2 expression levels were observed during postnatal development in the VTA such that increases in p-ERK1 and p-ERK2 levels are observed at PND7 and PND14 as compared to PND0 (Fig. 2). In these regions, the amount of ERK1 activation (*i.e.,* p-ERK1/ERK1) was significantly increased at PND7 and PND14, while the amount of ERK2 activation (*i.e.,* p-ERK2/ERK2) was significantly increased only at PND7 as compared to PND0 (Fig. 2). Overall, ERK5 expression and activation declined with postnatal development, whereas increases in ERK1 and ERK2 expression and activation were observed with development (Figs. 1 and 2).

3.2. ERK5, ERK1, and ERK2 levels in the striatum during postnatal brain development

ERK5 and p-ERK5 expression levels significantly decreased with postnatal development in the STR starting at PND7 and continuing to adult age as compared to PND0 (Fig. 3). Similar to ERK5 and p-ERK5 expression levels, significant decreases in p-ERK1 and p-ERK2 expression were observed with postnatal development at PND7 through adult age as compared to PND0 (Fig. 3). The amount of ERK1 and ERK2 activation (*i.e.,* p-ERK1/ERK1 and p-ERK2/ERK2) was also significantly decreased as compared to PND0 (Fig. 3). Although decreases in phosphorylated ERK1, ERK2, and ERK5 and total ERK5 expression were noted with postnatal development, a trend increase in ERK1 and a significant increase in ERK2 were observed with the highest expression observed at adult age (Fig. 3).

4. Discussion

In the current study, ERK1, 2, and 5 expression and activation were examined in the ventral mesencephalon and STR regions during postnatal development. To this end, the protein levels of total

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