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CHANGES IN SYNAPTIC TRANSMISSION AND PROTEIN EXPRESSION IN THE BRAINS OF ADULT OFFSPRING AFTER PRENATAL INHIBITION OF THE KYNURENINE PATHWAY

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Abstract—During early brain development, N-methyl-D-aspartate (NMDA) receptors are involved in cell migration, neuritogenesis, axon guidance and synapse formation, but the mechanisms which regulate NMDA receptor density and function remain unclear. The kynurenine pathway of tryptophan metabolism includes an agonist (quinolinic acid) and an antagonist (kynurenic acid) at NMDA receptors and we have previously shown that inhibition of the pathway using the kynurenine-3-monoxygenase inhibitor Ro61-8048 in late gestation produces rapid changes in protein expression in the embryos and effects on synaptic transmission lasting until postnatal day 21 (P21). The present study sought to determine whether any of these effects are maintained into adulthood. After prenatal injections of Ro61-8048 the litter was allowed to develop to P60 when some offspring were euthanized and the brains removed for examination. Analysis of protein expression by Western blotting revealed significantly reduced expression of the GluN2A subunit (32%) and the morphogenetic protein sonic hedgehog (31%), with a 29% increase in the expression of doublecortin, a protein associated with neurogenesis. No changes were seen in mRNA abundance using quantitative real-time polymerase chain reaction. Neuronal excitability was normal in the CA1 region of hippocampal slices but paired-pulse stimulation revealed less inhibition at short interpulse intervals. The amount of long-term potentiation was decreased by 49% in treated pups and recovery after low-frequency stimulation was delayed. The results not only strengthen the view that basal, constitutive kynurenine metabolism is involved in normal brain development, but also show that changes induced prenatally can affect the brains of adult offspring and those changes are quite different from those seen previously at weaning (P21). Those changes may be mediated by altered expression of NMDAR subunits and sonic hedgehog.

Key words: kynurenines, kynurenic acid, tryptophan, neurodevelopment, doublecortin, hedgehog.

INTRODUCTION

There is increasing interest in the role of epigenetic modification in the early development of the nervous system. A wide range of factors can modify brain development after fetal or early postnatal exposure, including diet, stress, therapeutically used medicines and environmental agents. There is relatively little information, however, on the mechanisms by which such external influences induce changes in brain development.

One possibility is that changes are induced in the activation of glutamate receptors for N-methyl-D-aspartate (NMDA), since these are known to be intimately involved in early phases of brain development. NMDA receptors play key roles in the initial formation and guidance of axon branches, the establishment and stabilization of synaptic contacts and the induction and maintenance of dendritic spines (Simon et al., 1992; Rajan and Cline, 1998; Ernst et al., 1998; Heng et al., 1999; Cuppini et al., 1999; Udin and Grant, 1999; Colonnese et al., 2005; Alvarez et al., 2007; Ultanir et al., 2007). These and other aspects of neuronal and synaptic development ultimately determine synaptic function and plasticity in the mature, postnatal, offspring (Iwasato et al., 2000; Myers et al., 2000; Ramoa et al., 2001; Fagiolini et al., 2003).

In addition, antagonists acting at NMDA receptors prevent many of these neuro-developmental processes and, when administered during late fetal or early postnatal life, increase the natural loss of neurons and synapses (Ikonomidou et al., 1999; Dikranian et al., 2001; Harris et al., 2003; Vincent et al., 2004).

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Abbreviations: aCSF, artificial cerebrospinal fluid; ANOVA, analysis of variance; CED, Cambridge Electronic Design; COX-2, cyclo-oxygenase-2; DISC-1, Disrupted in Schizophrenia-1; fEPSP, field excitatory postsynaptic potential; HRP, horseradish peroxidase; IDO, indoleamine-2,3-dioxygenase; KMO, kynurenine-3-monoxygenase; LTP, long-term potentiation; LTD, long-term depression; NMDA, N-methyl-D-aspartate; P, postnatal day; PCNA, Proliferating Cell Nuclear Antigen; PPF, paired-pulse facilitation; PPI, paired-pulse inhibition; PSD-95, Post-Synaptic Density molecule-95; qt-PCR, quantitative real-time polymerase chain reaction; Ro61-8048, 3,4-dimethoxy-N-[4-(3-nitrophenyl)thiazol-2-yl]-benzenesulfonamide; TBST, Tris-buffered saline containing 0.05% Tween; TH, tyrosine hydroxylase; VAMP-1, Vesicle-Associated Membrane Protein-1.

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The neuronal and synaptic disruption produced by exogenously administered NMDA receptor antagonists results in profound abnormalities of neuronal development, brain structure and behavior reminiscent of those seen in schizophrenia (Harris et al., 2003; du Bois and Huang, 2007). However, previous studies do not address the question of whether manipulating physiological, endogenous factors affecting NMDA receptor function could also result in disordered neuronal development.

One potential method for modulating the activation of NMDA receptors by endogenous ligands is to interfere with the kynurenine pathway. This pathway is the major route for the metabolism of tryptophan and generates quinolinic acid, a selective agonist at NMDA receptors (Stone and Perkins, 1981; Stone and Darlington, 2002) as well as kynurenic acid, which is an antagonist at all ionotropic glutamate receptors, though with greatest potency at NMDA receptors (Perkins and Stone, 1982; Stone et al., 2013). Kynurenic acid may also block nicotinic receptors in the CNS (Hilmas et al., 2001) although this has been disputed (Mok et al., 2009; Dobelis et al., 2012). The ratio between the endogenous levels of quinolinic acid and kynurenic acid will, therefore, influence neuronal excitability and viability.

In this study we have used an inhibitor of kynurenine-3-monoxygenase (KMO) to alter the relative concentrations of endogenous quinolinic acid and kynurenic acid. Previous work has shown that the major effect of inhibition by 3,4-dimethoxy-N-[4-(3-nitrophenyl)thiazol-2-yl]-benzenesulfonamide (Ro61-8048; Röver et al., 1997) is to raise the levels of kynurenic acid in the blood and brain (Röver et al., 1997; Cozzi et al., 1999; Clark et al., 2005; Forrest et al., 2013). The compound was administered to gestating female rats and the offspring were allowed to develop normally until day 60 (P60). The effects of the exposure to Ro61-8048 were then examined on hippocampal synaptic transmission and plasticity in the brains of those P60 animals. It is around this age that many studies have shown behavioral changes resulting from prenatal infection or mimetic agents (Iwasato et al., 2000; Fatemi et al., 2005; Zuckerman and Weiner, 2005) and we therefore tested behavior in the open-field test of exploration and a step-down inhibitory avoidance task of learning. Since many of the neurodevelopmental functions of NMDA receptors are probably mediated via their interactions with cell proliferation, axonal guidance and cytoskeletal molecules which are crucial to the development and maintenance of synaptic contacts (Ozaki et al., 2000; Hoffman et al., 2001; Ramoa et al., 2001; Fagiolini et al., 2003), we have also included an examination of the expression of representative proteins as well as the mRNA for key proteins by quantitative real-time polymerase chain reaction (qRT-PCR). The target molecules examined are all known to play key roles in neuronal development, neurite outgrowth, synapse formation and neurotransmitter release. They include components of the synaptic vesicle and neurotransmitter release machinery such as synaptophysin, Vesicle-Associated Membrane Protein-1 (VAMP-1; synaptobrevin) and the vesicular release calcium sensor synaptotagmin. As markers of axon guidance and synapse formation, the proteins EphA4, Unc5H1 and Unc5H3 were studied, in addition to the cytoskeletal modulators RhoA and RhoB, the glutamate complex component Post-Synaptic Density molecule-95 (PSD-95) and tyrosine hydroxylase (TH).

We have also examined the expression of two proteins that might be important indicators of the basic mechanisms underlying the observed changes: the interneuronal maturation molecule doublecortin and the morphogenetic protein sonic hedgehog.

Finally, whereas the juvenile (P21) study was performed on whole cerebral hemispheres, the present, more detailed analysis of adults at P60 was focussed on the hippocampus.

EXPERIMENTAL PROCEDURES

This study was carried out according to the regulations of the Animals (Scientific Procedures) act 1986 of the UK, administered and monitored by the Home Office. Male and female Wistar rats were housed together for mating and inspected daily for the occurrence of a vaginal plug. Thereafter, the pregnant females were housed alone with free access to food and water.

To inhibit tryptophan oxidation along the kynurenine pathway we used Ro61-8048 (Röver et al., 1997). This compound is an inhibitor of KMO, a key enzyme in the pathway which shifts the balance of tryptophan metabolism away from the generation of the NMDA receptor agonist, quinolinic acid, toward the antagonist, kynurenic acid (Cozzi et al., 1999; Clark et al., 2005; Forrest et al., 2013). From those earlier studies we identified the dose of 100 mg/kg (i.p.) as one which can be administered repeatedly to the same animal (Clark et al., 2005; Rodgers et al., 2009). In order to maximize the period of development during which the activity of the kynurenine pathway is affected, we administered this compound to the pregnant dam at days E14, E16 and E 18 of gestation. Groups of control animals were injected with the saline vehicle. In most experiments, gestation was allowed to proceed normally, with neonates being removed from the home cage for euthanasia followed by removal of the brain when adult at postnatal day (P60). In an earlier study of postnatal animals at the time of weaning (P21) we confirmed that prenatal exposure to Ro61-8048 had the predicted effects on levels of kynurenine and kynurenic acid, concentrations of both being increased between 10 and 100-fold in the maternal blood and embryo brains (Forrest et al., 2013).

Electrophysiology

Electrophysiological studies were performed on male and female animals which were allowed to wean and grow under normal conditions to 60 days of age (P60 animals) with food and water available *ad libitum*. Animals were killed by administration of an overdose of