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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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Highlights

- An inhibitor of kynurenine-3-monoxygenase was administered to pregnant female rats

- Adult offspring at postnatal day 60 (P60) showed alterations in synaptic plasticity

- Expression of GluN2A, sonic hedgehog and other developmental proteins were changed

- Most earlier protein changes had disappeared with no changes in RNA or learning tasks

- Prenatal kynurenine pathway inhibition produces persistent synaptic and protein changes

Abstract

During early brain development, 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 euthanised 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 expression of doublecortin, a protein associated with neurogenesis. No changes were seen in mRNA abundance using qRT-PCR. 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.

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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 foetal 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 (Heng et al., 1999; Cuppini et al., 1999; Rajan & Cline 1998; Colonnese et al., 2005; Ernst et al., 1998; Simon et al., 1992; Udin & Grant 1999; 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 (Fagiolini et al., 2003; Iwasato et al., 2000; Myers et al., 2000; Ramoa et al., 2001).

In addition, antagonists acting at NMDA receptors prevent many of these neuro-developmental processes and, when administered during late foetal or early postnatal life, increase the natural loss of neurons and synapses (Dikranian et al., 2001; Ikonomidou et al., 1999; Vincent et al., 2004; Harris et al., 2003). The neuronal and synaptic disruption produced by exogenously administered NMDA receptor antagonists results in profound abnormalities of neuronal development, brain structure and behaviour reminiscent of those seen in schizophrenia (Harris et al., 2003; du Bois & 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 & Perkins 1981; Stone & Darlington 2002) as well as kynurenic acid, which is an antagonist at all ionotropic glutamate receptors, though with greatest potency at NMDA receptors (Perkins & 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]-benzenesulphonamide (Ro61-8048; Rover et al., 1997) is to raise the levels of kynurenic acid in the blood and brain (Rover 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 behavioural changes resulting from prenatal infection or mimetic agents (Fatemi et al., 2005; Iwasato et al., 2000; Zuckerman & 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 (Fagiolini et al., 2003; Hoffman et al., 2001; Ramoa et al., 2001; Ozaki et al., 2000), we have also included an examination of the expression of representative proteins as well as the mRNA for key proteins by 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 3,4-dimethoxy-N-[4-(3-nitrophenyl)thiazol-2-yl]benzenesulphonamide (Ro61-8048) (Rover et al., 1997). This compound is an inhibitor of kynurenine-3 monoxygenase (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, towards 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 100mg/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 maximise 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 urethane (2.5g /kg rat delivered as an i.p. injection of a 25% solution in water) followed by cervical dislocation. The brain was removed into ice-cold artificial cerebrospinal fluid (aCSF) of composition: (in mM) NaCl 115; KH_2PO_4 2.2; KCl 2; $MgSO_4$ 1.2; $NaHCO_3$ 25; $CaCl_2$ 2.5; glucose 10, gassed with 5%CO_2 in oxygen. The hippocampi were rapidly

removed and chopped into 450μm transverse slices using a McIlwain tissue chopper. The slices were preincubated at room temperature for at least 1 hour in a water-saturated atmosphere of 5%CO_2 in O_2 before individual slices were transferred to a 1 ml capacity superfusion chamber for recording. Slices were superfused at 28-30°C using aCSF at a flow rate of 3-4 ml/min. A concentric bipolar electrode was used for stimulation of the Schaffer collateral and commissural fibres in stratum radiatum, using stimuli delivered at 0.1 Hz or 0.05 Hz with a pulse width of 50-300 μs, adjusted to evoke a response amplitude of approximately 70% of maximum to allow increases or decreases in size to be detected. Extracellular recordings were made via glass microelectrodes containing 1M NaCl (tip diameter approximately 2μm, DC resistances 2-5MΩ) with the tip positioned under microsopic visualisation in the stratum radiatum of the CA1 region to evoke field excitatory postsynaptic potentials (fEPSPs). Potentials were amplified, digitised and stored on computer via a CED (Cambridge Electronic Design, Cambridge, UK)) micro1401 interface. The fEPSPs were routinely quantified by measurement of the early positive slope of the potential, using Signal software (CED, Cambridge, UK). The axonal volley was monitored wherever it was possible to distinguish it clearly from the fEPSP in order to ensure that no change occurred during the experiments.

Once placed into the recording chamber, the recording of fEPSPs was allowed to stabilise and a minimum period of 10min obtained at a stable baseline. The degree of LTP and LTD was quantified by measuring the size of the evoked potentials once a post-stimulation plateau had been obtained and comparing with the size of the potential before stimulation.

Paired-pulse interactions were assessed in slices not used for the examination of LTP or LTD, using pairs of stimuli S1 and S2 with inter-stimulus intervals of 10 -100ms. In the case of fEPSPs the 10ms interval resulted in a substantial overlap between successive potentials and an electronic subtraction was performed in which a single evoked potential

at time S1 was subtracted from a subsequent paired-pulse response to reveal the true magnitude of the response to S2.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated from snap frozen, whole hippocampi removed from P60 rats (as previously described, n = 3/litter, n = 3 litters/treatment group, n = 9 control and n = 9 treated) using the Qiagen RNeasy lipid mini kit with DNaseI treatment, according to the manufacturer's instructions. The RNA concentration of each sample was determined using a NanoDrop (Thermo Scientific) and the integrity of each mRNA sample was assessed using the Agilent 2100 Bioanalyser (Glasgow Polyomics Facility, University of Glasgow). Only samples with RNA integrity number (RIN) values greater than 8 were used (values ranged from 8.70 - 9.30).

First strand cDNAs were synthesised from total RNA using random hexamers and Superscript III Reverse transcriptase in the VILO cDNA synthesis kit (Life Technologies) following the manufacturer's instructions.

qRT-PCR was carried out in an ABI cycler (SDS7000) using the TaqMan Gene expression assay system for the following assays: doublecortin (*Dcx*; Rn00670392_m1), sonic hedgehog (*Shh*; Rn00568129_m1), NMDA receptor subunit 2A (*Grin2a*; Rn00561341_m1) and NMDA receptor subunit 2B (*Grin2b*; Rn00680474_m1), (Applied Biosystems). In addition, four reference genes were used: beta actin (*Actb*; Rn00667869_m1), glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*; Rn01775763_g1), beta-2 microglobulin (*B2m*; Rn00560865_m1) and 18S ribosomal RNA (*Rn18s*; Mm03928990_g1), (Applied Biosystems).

Immunoblotting

Brain sample homogenates were prepared in RIPA buffer (50mM Tris, 150mM NaCl, 0.1% SDS, 0.5% Triton X-100, 1% IGEPAL, and a Roche complete protease inhibitor tablet) and centrifuged at 18000 g for 5 min at 4°C. Supernatants were collected for protein concentration determination using the Bio-Rad Coomassie Blue protein assay (Bio-Rad, Hemel Hempstead, UK). Samples were then normalised to 10μg and prepared as; 65% protein sample, 25% sample buffer and 10% reducing agent (Life Technologies, Paisley, UK) and heated at 70°C for 10 min. The protein samples were loaded onto NuPAGE Novex 4-12% Bis-Tris (1.0mm) 15 lane gels (Life Technologies, Paisley, UK) and run at 150 volts for 80 min to separate proteins according to their molecular weight. SeeBlue pre-stained standard (Life Technologies, Paisley, UK) was included on each gel as a molecular weight marker. The separated proteins were then blotted onto Invitrolon poly(vinylidene difluoride) membranes (Life Technologies, Paisley, UK) at 30V for 60 min. The membranes were blocked for 1h in 5% non-fat dried milk solution in Tris-buffered saline containing 0.05% Tween (TBST) before overnight incubation at 4°C with the appropriate primary antibody (diluted in 5% milk-TBST). Membranes were then washed 3 times for 15 min with TBST and incubated with the appropriate horseradish peroxidase (HRP) conjugated secondary antibody (prepared in 5% milk-TBST) for 1h at room temperature. Following secondary antibody incubation, blots were washed 3 times for 15 min with TBST then visualised using Enhanced Chemiluminescence Plus detection kit (GE Healthcare, Chalfont St Giles, UK).

Western blot analysis was carried out using the following primary antibodies:-
From Millipore, Watford, UK:- GluN1 (mouse monoclonal, 05-432, 1 : 1000 dilution); tyrosine hydroxylase (mouse monoclonal, MAB5280, 1 : 10000 dilution), synaptophysin (mouse monoclonal, MAB368, 1 : 40000 dilution)

From R&D Systems, Abingdon, UK:- GluN2A (rabbit polyclonal, PPS012, 1:5,000 dilution);

GluN2B (rabbit polyclonal, PPS013, 1:5,000); VAMP-1/synaptobrevin (goat polyclonal,

AF4828, 1 : 10,000 dilution), synaptotagmin (MAB 43641, 1:5,000 dilution);

From Cell Signalling, New England Biolabs, Hitchin, Herts, UK:- PSD-95 (rabbit

monoclonal, #3450, 1:10,000 dilution);

From Santa Cruz, Insight Biotechnology, Wembley, UK:- doublecortin (goat polyclonal, sc-

8066, 1:1000 dilution); α-synuclein (mouse monoclonal, sc-65500, 1:1000 dilution); actin

(goat polyclonal, sc-1615, 1:10,000 dilution); DISC-1 (goat polyclonal, sc-47990, 1:1000

dilution); Unc5H1 (goat polyclonal, sc-67902, 1:1000 dilution), Unc5H3 (goat polyclonal,

sc-54442, 1:1000 dilution), SHH (goat polyclonal, sc-1194, 1:1000 dilution); NFκB (rabbit

polyclonal, sc-372, 1:5,000 dilution); COX-2 (goat polyclonal, sc-1745, 1:1000 dilution);

RhoA (mouse monoclonal, sc-418, 1:1,000 dilution); RhoB (mouse monoclonal, sc-8048,

1:1000 dilution); EphA4 (rabbit polyclonal, sc-921, 1:5000); PCNA (mouse monocloncal,

sc-56, 1:1000 dilution; 5HT-2c (mouse monoclonal, sc-17797, 1:1000 dilution)

   The following secondary HRP-conjugated antibodies were used at 1 : 5000 dilution:

goat anti-rabbit HRP (12-348) (Millipore, Watford, UK); donkey anti-goat HRP (sc-2020)

and goat anti-mouse (sc-2005) (Santa Cruz, Insight Biotechnology, Wembley, UK)

Behaviour

   All experiments were performed in accordance with the Review Committee of the

Veterinary School (CICUAL), University of Buenos Aires and the International Brain

Research Organization (IBRO), and are in compliance with the U.S. National Institutes of

Health Guide for Care and Use of Laboratory Animals (publication no. 85-23, revised

1985) and the European Communities Council Directive of 24 November 1986

(86/609/EEC).

Wistar rats were treated as previously described (see Methods above); pregnant Wistar rats were injected i.p. either with Ro61-8048 or the saline vehicle. At P21 female and male rats (35-40 g) bred in-house were separated and maintained in groups of 5-6 per cage, under a 12 h light/dark inverted cycle with water and food available *ad libitum* until P60 (180-220g).

Open field test of locomotor and exploratory activities

To evaluate possible changes of locomotor activity and/or exploratory behaviour, P60 female and male rats – born from mothers treated with Ro61-8048 or saline - were initially allowed to freely explore an open square field (measuring 60.0 cm long × 60.0 cm wide × 50.0 cm high) for 5 min (OF1). The floor of this arena was divided into 16 sectors of 15.0 cm × 15.0 cm each, and the number of rearings, groomings and crossings from one sector to another per minute were counted in the initial 5 minutes session (OF1) (carried out at about 3 h before avoidance training); the second 5 minutes session (OF2) was performed in the following day, about 2 h after the avoidance test, between 27 and 28 h from the first OF session.

Step-down inhibitory avoidance

Effects on learning and memory were tested using a step-down inhibitory avoidance model of learning in rats as described previously (Harvey et al., 2012).

Rats were trained in the step-down inhibitory avoidance task at about 3 h after the first open field session. A rat was placed on an elevated isolated platform (25 x 7 x 2.5 cm high) at the left side of an acrylic box (50 x 25 x 25 cm), with the floor made of parallel bronze bars (0.5 cm calibre, 0.5 cm apart). The latency was measured for the animal to step-down from the platform, placing all four paws on an electrifiable grid (training latency), at which point the animal was given a 2.0 sec, 0.75 mA scrambled foot-shock, causing it to

return to the platform or remain on the grid from which it was immediately removed and returned to its home-box.

In the next day (about 20 h after the training session), a retention test was performed in which the step-down latency (test latency) was recorded up to a maximum of 300 sec, but no shock was delivered. The difference between test and training latencies was taken as an indication of retention: a better memory should result in a higher test latency and a greater difference from the training latency

Data analysis and statistics

Immunoblotting: All western blots were quantified using the Image J software (http://rsb.info.nih.gov/ij/) and comparisons were made statistically between groups of pups born to mothers treated with Ro61-8048 and groups born to mothers injected with saline vehicle. This protocol allowed the use of a *t*-test to examine differences between the two groups. To control for variations in the total amount of protein loaded onto gels all samples were examined after staining with Ponceau S stain. In addition, actin levels were examined in each series of blots and the ratio taken of the intensity of target protein to the intensity of actin. A probability value of 0.05 was adopted as the criterion for significance. Actual p-values are indicated when >0.0001.

Electrophysiology: Data from hippocampal slices are presented as mean ± standard error. Baseline values were obtained from a stable 10 min period of evoked potential size prior to the addition of any drugs, with the first of those potentials being defined as 100%. This allowed the 10 min pre-drug period to provide an indication of baseline variance. Repeated measures ANOVA were used for statistical comparisons followed by the Bonferroni *post hoc* test for individual comparisons.

qRT-PCR: PCR Ct values and reaction efficiencies were obtained from the raw fluorescent data using LinReg PCR (Ramakers *et al.*, 2003). TaqMan assay reaction

efficiencies were *Dcx* 1.802, *Shh* 1.876, *Grin2a* 1.858, *Grin2b* 1.866, *Actb* 1.807, *Gapdh* 1.729, *B2m* 1.866 and *Rn18s* 1.781. Samples were run in triplicate and Ct outliers were determined using the Grubb's test http://graphpad.com/quickcalcs/Grubbs). Significant Ct outliers (P>0.05) were removed before statistical analysis. The mean Ct values for each sample were analysed using REST 2009 v2.0.13 (Pfaffl *et al.*, 2002). REST calculates P values on the basis of 2000 permutations of a pair-wise fixed reallocation randomisation test in which Ct values for the reference and target gene are reallocated to sample and control groups and calculates the resulting expression ratios on the basis of the mean values.

Behaviour. For the behavioural tasks, non-parametric statistics were used because an upper time limit (300s) was specified for stepping-down from the platform in the avoidance task. Results are presented as medians with inter-quartile ranges (Q_{25} / Q_{75}). Statistical differences between test latencies were evaluated using a Kruskal-Wallis ANOVA. When significant differences were found, training and test latencies in each group were compared by the Wilcoxon test. Latencies for both training and test sessions, and differences between them (test minus training latencies) were compared between groups (Ro61-8048 vs. saline) using the Mann-Whitney 'U' test, to evaluate effects of drug treatment.

Data from the open-field exploration task are expressed as the medians of the number of crossings or rearings performed during a 5 min session (OF1 or OF2) by animals exposed prenatally to vehicle (OF1 control and OF2 control) or Ro61-8048 (OF1 treated and OF2 treated). Groups were compared using Kruskal-Wallis ANOVA. When significant differences were found, the numbers of crossings or rearings in each group were compared by the Wilcoxon test. Further comparison between groups (Ro61-8048 vs. vehicle) was made using the Mann-Whitney 'U' test.

Results

Electrophysiology

Initial experiments on each hippocampal slice established the absolute levels of excitability by measuring the size of the fEPSP in stratum radiatum as the stimulus was increased from the threshold potential that elicited a discernible response (Fig. 1A). The slopes of the potentials were compared up to the stimulus level at which the first sign of a population spike indentation appeared in the fEPSP. There were no significant differences between any of the data points in the stimulus-response relationship between slices prepared from control animals and those from animals born to dams treated with Ro61-8048 (Fig. 1A).

A similar analysis was performed for population spike amplitude (Fig. 1B), but no significant difference was detected between the stimulus response curves for control and Ro61-8048-exposed animals.

In order to obtain more detailed information about the pre- and post-synaptic function of synapses in these animals, pairs of stimuli were used, at interpulse intervals ranging from 100ms (10Hz) to 10ms (100Hz), to examine the occurrence and magnitude of paired-pulse facilitation (PPF) and paired-pulse inhibition (PPI). At the 10ms interval there was too much overlap between the evoked fEPSPs to allow a direct comparison of the first (fEPSP1) and second (fEPSP2) potentials, and the Signal software was used to perform a digital subtraction of fEPSP1 from the paired-pulse potentials (Fig. 1Ca-e). While the overall profile of the potential ratios at varying interpulse intervals are similar between slices taken from control and Ro61-8048-treated animals, there was a significant difference between the fEPSP responses of the slices at the shortest interpulse interval of 10ms (Fig. 1C).

Digital subtraction was not necessary for the analysis of population spikes, for which the relatively rapid time course of the spike, together with the much greater degree of facilitation or inhibition, made the contribution of any overlap negligible. A similar relationship was found to that observed with fEPSPs, although control slices showed a significant degree of PPI at the 10ms interpulse interval as exemplified by the potentials illustrated in Fig. 1Da-d). In contrast, slices from Ro61-8048-exposed animals showed less facilitation at the 10ms interval compared with longer interpulse intervals, but no overall PPI, leading to a highly significant difference compared with the controls animals (Fig. 1D), and an exaggeration of the difference noted above with fEPSPs.

However, it is recognised that, primarily at smaller interpulse intervals, the nature of the interaction between potential pairs of population spikes is highly dependent on the frequency with which the pulse pairs are elicited. In view of the difference between the test and control slices at the 10ms interpulse interval, therefore, this relationship was examined further. It was found that, at increasing frequencies of paired-pulse presentation, control slices showed little change in the degree of PPI seen at 10ms (Fig. 1C,E), whereas in slices from Ro61-8048-treated animals the initial PPF was lost over a series of 5-10 stimulus pairs, leaving PPI at higher presentation frequencies as illustrated in the potentials of Fig. 1Ea,b. The switch from facilitation to inhibition was sufficiently pronounced that a very significant difference was demonstrated at a paired-pulse frequency of 1Hz (Fig. 1E) as well as at the lower frequencies of 0.1 and 0.05Hz.

Long-term potentiation

Two aspects of synaptic plasticity were examined in the slices - long-term potentiation (LTP) and long-term depression (LTD). LTP was tested using a burst of theta stimulation (5 bursts per second, for 2 seconds, of 4 stimuli delivered at 100Hz; Larson et al., 1986). In control slices this level of stimulation induced an increase of fEPSP amplitude which

reached a plateau after approximately 20min and was stable until the end of the recording period at 50min (Fig. 2A,B). In order to compare potential size at the plateau with their pre-stimulation amplitude an analysis of variance was performed using the fEPSP slopes recorded over the 10min period before stimulation and between 40 to 50min after theta stimulation. In control animals this increase was statistically significant ($F_{[19,80]}$ = 17.462; p < 0.0001, n = 5) with an increase of 34.8% between the mean fEPSP slope at 0min (98.1 ± 3.5%) and that at 50min (133.0 ± 5.8%; p = 0.0009, n = 5).

For slices prepared from animals subjected to Ro61-8048 administration *in utero*, a very similar temporal profile of LTP was obtained, but of a lower magnitude than in control slices (Fig. 2A,C). Analysis of variance confirmed that the fEPSP slopes were still significantly greater than the corresponding pre-stimulation level ($F_{[19,80]}$ = 2.248; p = 0.0066, n = 5) with an increase of 17.7% between the mean fEPSP slope at 0min (97.3 ± 2.5%) and that at 50min (115.0 ± 7.4%, p = 0.05, n = 5). A comparison of the fEPSP slopes of control and Ro61-8048-exposed slices between 45 and 50min after stimulation indicated that the LTP was very significantly smaller in treated animals compared with controls ($F_{[19,80]}$ = 4.98, p < 0.0001, n = 5) (Fig. 2A).

Long-term depression

Previous studies of LTD in this laboratory have indicated that a clear depression of fEPSP slope can be obtained using a modification of the protocol published by Kemp and Bashir (1999). Stimulus trains of 3 pulses at 200Hz were delivered every second for 5mins (stim1, Fig. 2D), and the same protocol repeated 20mins later (stim2). In slices from control animals, a clear LTD was obtained in which the fEPSP slope between 30-40min after stim2 was very significantly lower than in the 10min baseline period preceding stim1 ($F_{[19,80]}$ = 6.94, p<0.0001, n = 5 slices), with a reduction of 32.0% between the fEPSP at 0min immediately preceding stim1 (102.7 ± 6.1%) and the fEPSP at 40min following stim2

(69.7 ± 5.6%; p = 0.004, n = 5) (Fig. 2D,E). In comparison, the slices taken from animals exposed to Ro61-8048 showed a similar degree of LTD such that the potential size between 30-40 min after stim2 was reduced very significantly ($F_{[19,80]}$ = 16.25, p<0.0001, n = 5 slices) with a reduction of fEPSP slope by 42.3% between the potentials at 0min preceding stim1 (105.2 ± 3.1%) and the mean potential recorded 40min after stim2 (63.0 ± 2.1%; p < 0.0001, n = 5) (Fig. 2D,F). The level of LTD between 30-40min after stim2 was not significantly different between slices from control animals and those exposed to Ro61-8048 (ANOVA, $F_{[19,80]}$ = 0.106, p > 0.99.)

There was, however, a difference in the time course of LTD development in the two groups (Fig. 2D). Slices from Ro61-8048-treated animals recovered more slowly from stim2 stimulation, and this was borne out by a comparison of the individual fEPSP slopes between 30 and 50min (0-20min after stim2). All points in this period between 31 and 37min were significantly different at p < 0.001 (two-tailed t test, n = 5), while points between 38 and 40 mins were different at p<0.01.

Adenosine sensitivity

While LTP and LTD are primarily dependent on postsynaptic phenomena, adenosine acts on A1 receptors located almost exclusively on presynaptic terminals, thus providing a relatively selective test of presynaptic function (Goncalves and Queiroz, 2008; Stone et al., 2009). In order to determine whether Ro61-8048 treatment had produced any change specifically in presynaptic function, therefore, slices were exposed to 10 and 30μM concentrations of adenosine perfused for 10min. This procedure reduced the slope of the fEPSP to a plateau level approximately 25 and 75% respectively below the baseline potential size in control animals (Fig. 3A-C). Using slices from animals exposed to Ro61-8048, a similar degree of reduction was obtained, the magnitude of the responses to

adenosine being not significantly different from controls at either concentration tested (Fig. 3D).

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

REST 2009 was used to analyse relative gene expression. *B2m* expression was significantly reduced in the Ro61-treated rats (p = 0.009). This reference gene was therefore excluded from further analysis. The reference genes used to analyse relative gene expression were *Actb, Gapdh* and *Rn18s* as these were not significantly different between control and treated rats. mRNA expression of *Dcx, Shh, Grin2a* and *Grin2b* transcripts did not change relative to *Actb, Gapdh* and *Rn18s* (Fig. 4).

Protein expression

The range of proteins examined in this study was similar to that targetted in our previous study of animals at 21 days of age (Forrest et al., 2013). They included proteins known to be involved in neuronal migration, early axon guidance, dendritogenesis and the formation of spines and synaptic contacts. Significant differences were noted in the expression of three of these proteins, in marked contrast to the variety of changes seen at P21, where 9 of 20 proteins examined showed significant changes of expression.

In several cases proteins which had shown no change at P21 remained unchanged at P60. These included the presynaptic vesicular release protein synaptophysin (p = 0.57), the vesicle release calcium sensor synaptotagmin (p = 0.14) and Vesicle Associated Membrane Protein-1 (VAMP-1; synaptobrevin) (p = 0.41). There was also no evidence of change for the dependence receptors Unc5H1 (p = 0.24) and Unc5H3 (p = 0.29), which are dependent on the secreted paracrine protein family of netrins and which play crucial roles in establishing the balance between attraction and repulsion between growing axons, dendrites and postsynaptic targets. Similarly, the pro-inflammatory transcription factor

NFκB as well as cyclo-oxygenase-2 (COX-2) - an important enzyme in generating the oxidative stress that accompanies tissue inflammation – were not different between test and control animals (p = 0.25 and p = 0.43 respectively).

In view of the role of NMDAR receptors for both synaptic development and function, particular importance was attached to the expression of the major receptor subunits especially since, at P21, there had been no change in GluN1 but very significant changes in the expression of both GluN2A and GluN2B subunits. In the present study, expression of the GluN2A subunit was significantly lower in P60 offspring exposed *in utero* to Ro61-8048 compared with controls (Fig. 5A) and this was a decrease in expression whereas levels were increased at P21. No difference was detected in expression of GluN2B or GluN1 subunits (Figs. 5B, 5C).

The levels of several proteins with strong links to NMDA receptor function were examined but neither the GluN1 subunit (p = 0.20, Fig. 5C) nor postsynaptic density protein-95 (PSD-95)(p = 0.14, Fig. 5D) showed a clear change even though the latter had been significantly increased at the P21 age point. The two small GTPase enzymes RhoA and RhoB, which are involved in some of the plastic phenomena triggered by NMDA receptors (O'Kane et al., 2003) were similarly unchanged (p = 0.95, Fig. 5E; p = 0.31, not shown, respectively) even though there had been a very significant increase in RhoB at P21.

In other cases, such as the axon guidance and synapse formation receptor EphA4 (p = 0.70, Fig. 5F) and Proliferating Cell Nuclear Antigen (PCNA, p = 0.19, not shown) changes seen at P21 had been resolved or compensated so that no significant difference was apparent by the age of P60.

There were, however, marked and highly significant differences in the expression of two of the proteins concerned with cell proliferation, differentiation and maturation, and which had shown altered expression in animals at the time of weaning (P21). Doublecortin, which

is associated with the undifferentiated state of cells and declines with differentiation, exhibited a clearly significant alteration in the pups exposed to Ro61-8048, with a 30% increase in expression compared with saline-treated animals ($p = 0.03$; Fig. 5G). This was very similar to the 30% increase seen in animals at P21.

The sonic hedgehog protein (Shh), was clearly detected in the adult hippocampus. Expression of this molecule was decreased significantly in the Ro61-8048-treated animals compared with controls ($p = 0.05$; Fig. 5H), a result again very close to that seen at P21.

Lastly, four proteins with a major relevance to dopaminergic neuronal systems were examined in this study: tyrosine hydroxylase (TH, $p = 0.58$), α-synuclein ($p = 0.52$), Disrupted in Schizophrenia-1 (DISC-1, $p = 0.89$) and the $5HT_{2C}$ receptor ($p = 0.58$), but none of these showed any change between treated and control animals in the hippocampus.

Behaviour

Open field exploration

Tests were performed on P60 rats after prenatal maternal administration of Ro61-8048. Animals were exposed twice to an open field arena on two consecutive days. As indicated in Fig. 6, there were no significant differences between the performance of control ($n = 38$) and Ro61-8048-treated ($n = 53$) offspring on their first exposure (OF1) to the arena, assessed either as the total number of line crossings (Fig. 6A) or the number of rearings in the 5min session (Fig. 6B) (OF1; $p > 0.05$, Mann-Whitney test). When tested in the arena at about 20 h later (OF2), both groups of animals showed a significant degree of habituation learning with fewer line crossings and rearings (Figs. 6AB) ($p < 0.05$, Wilcoxon signed rank test after Kruskal–Wallis ANOVA for non-parametric samples). Furthermore, the variables were recorded and compared every minute in the open field to assess short term recognition of the environment. In all groups the exploratory parameters decreased

during the session confirming that short term habituation to the arena was also preserved (Kruskal-Wallis followed by Dunn's multiple comparison test, not shown).

Step-down avoidance

In the inhibitory step-down avoidance task, animals in the control (n = 34) and treated (n = 50) groups showed a significant difference between latencies in training (TR) and test (TEST) sessions, the test latencies being significantly lower (Fig. 7A; Wilcoxon test), indicating that both groups learned the avoidance task and remembered it in the testing session 24 h later. There was no significant difference between the control and test groups in their response latencies in either the training or test sessions, indicating that Ro61-8048 had not affected the initial acquisition or the subsequent memory consolidation and further recall of the task.

In order to exclude any confounding effect of gender factors on these results, a separate analysis was performed of male (Fig. 7B; control n = 20, treated n = 19) and female (Fig. 7C: control n = 14, treated n = 31) animals. Although female rats showed greater variability in their response latencies, there remained no significant differences between control and treated groups.

Discussion

We have reported previously that the administration of Ro61-8048 to pregnant rats rapidly produces a substantial increase of between 10 and 100-fold in the levels of kynurenine and kynurenic acid in the maternal blood and in the embryo brains within 5hours (Forrest et al. 2013). After 24hours the maternal levels have normalised whereas they remain significantly elevated in the embryonic brain. It is likely, therefore, that the changes described previously at P21 and reported here for animals at P60 following the

same prenatal injection protocol result from the increased levels of these tryptophan metabolites. It is unlikely that changes were produced in the concentrations of quinolinic acid since previous work did not reveal any change in resting levels of this compound after Ro61-8048 (Chiarugi & Moroni, 1999; Clark et al., 2005).

It was also reported that the use of Ro61-8048 was not accompanied by any changes in the levels of pro-inflammatory cytokines such as interleukin-1α, IL-1B, IL-6, tumour necrosis factor-α, monocyte chemoattractant protein-1, macrophage inflammatory protein-1α, Regulated on Activation, Normal T cell expressed and secreted (RANTES), or interferon receptor (Forrest et al. 2013), confirming that activation of immune function was unlikely to contribute significantly to the observed results.

Electrophysiology

While the various biochemical changes generated in the adult brain by exposure to Ro61-8048 in utero indicate that abnormalities of neurochemistry can be produced by changing the kynurenine environment during early brain development, those changes may not be sufficient to cause gross functional change. In order to assess the existence and degree of functional change we have analysed several basic aspects of hippocampal electrophysiology.

The results indicate that there are no apparent alterations to basic aspects of neuronal excitability and synaptic transmission since there were no differences between control and treated animals in the stimulus-response relationships for either fEPSPs or population spikes. These data also imply that there are no changes in the relationship between presynaptic transmitter release and postsynaptic excitability (E-S coupling).

There are, however, clearly demonstrable changes in some aspects of synaptic plasticity. While slices from control and treated animals exhibit closely similar PPF across the range of interpulse intervals from 20 to 100ms, marked differences are apparent at the

10ms interval. The importance of this observation lies in the different mechanisms underlying PPF and PPI. The latter is believed to result from the short-lived depletion of synaptic vesicles following the first stimulus (Rosenmund and Stevens, 1996; Wang and Kaczmarek, 1998), whereas PPF is believed to result from the accumulation of residual calcium within synaptic terminals, leading to increased transmitter release and an increased size of the second potential of the pair (Zucker et al., 1991). A reduced, or absent, PPI at the smallest interpulse interval would, therefore, suggest less vesicular depletion, perhaps implying either an increased density of vesicles available for release, or a facilitated movement of vesicles to the active zone for release. The overall result would be an increased probability of vesicular release. It is also possible that the accumulation of intra-terminal calcium begins to occur earlier in synaptic terminals from treated animals so that the balance of mechanisms causing PPI and PPF is altered at this earliest time interval.

Another possibly relevant factor stems from the observation by Al-Hayani and Davies (2002) that the occurrence of secondary spikes predisposes slices to generate relatively weak PPI. However, care was taken in this study to examine only those slices which did not exhibit a secondary spike, making this factor an unlikely complication.

The occurrence of altered synaptic behaviour was strongly supported by extending this analysis to an examination of paired-pulse interactions at different frequencies of paired-pulse delivery. Increasing the frequency of stimulation from 0.05 to 1Hz has been shown previously to induce a shift from PPF to PPI in the hippocampus (Saviane et al. 2002). A similar change from PPF to PPI was reported following exposure to 4-aminopyridine, a potassium channel blocker which facilitates transmitter release (Pena et al., 2002) while increased paired-pulse inhibition was reported on exposure to bicuculline (Higgins and Stone, 1993) or following seizure activity (Stringer and Lothman, 1989). It has also been

reported that a similar shift from PPF to PPI can be induced by raising the stimulation strength (Huang et al., 2007).

In the present study, increasing stimulation frequency had little effect on the PPI seen in control slices, whereas the early PPF seen in slices from experimental animals was converted to PPI which, at a frequency of 1Hz, significantly exceeded the magnitude of PPI in control slices. Together, these various paired-pulse results suggest a facilitated release of transmitter in treated animals at small interpulse intervals, with either an increased depletion of vesicles at higher pair frequencies or a reduction in the opposing accumulation of calcium, which normally maintains transmitter release approximately constant over the frequency range examined.

There are, however, other factors that need to be examined in further detail in future work, since it has been suggested that presynaptic inhibitory receptors (Forsythe and Clements, 1990) including those for adenosine (Higgins and Stone, 1996), receptor desensitisation (Otis et al., 1996; Neher and Sakaba, 2001) or some form of refractory state (Waldeck et al., 2000) may contribute to paired-pulse interactions. GABA receptors may also be involved (Nathan et al., 1990) although this is likely to affect multiple spiking rather than primary potentials (Leung and Fu, 1994) and care was always taken in this study to limit investigation to slices showing no secondary spikes following single stimuli. While there is relatively little evidence that these processes contribute significantly to paired-pulse phenomena under normal conditions, it is of course possible that they may assume greater importance in the animals exposed to Ro61-8048.

Finally, changes were sought in the amplitude of LTP and LTD, the former induced by theta-burst stimulation which reproduces natural patterns of hippocampal activity and produces an optimal level of LTP (Larson et al., 1986). Exposure to Ro61-8048 in utero led to a defect in theta-burst LTP which, while still occurring to a significant extent, was significantly smaller than in slices from control animals. The specificity of the phenomenon

was emphasised by the absence of any significant difference in the amplitude of LTD, although the significant differences described in the time course of recovery from the second period of stimulation might reflect a degree of functional synaptic change. Indeed, both the reduced LTP amplitude and the slowed recovery towards the plateau LTD could be accommodated broadly within a hypothesis that explained the increased ratio of inhibition to facilitation at the higher frequencies of paired-pulse delivery. It is interesting to note a recent report that the expression of GluN2A, but not of GluN2B, was increased in the rat hippocampus 1 h after LTP induction (Baez et al., 2013). Hence, the decrease in LTP reported here may be, at least in part, a consequence of the reduced expression of GluN2A in the offspring from Ro61-8048-treated rats.

Mechanisms underlying altered development

It is well established that neuronal receptors for glutamate and acetylcholine are heavily involved in brain development. Glutamate receptors, especially those sensitive to NMDA affect neuronal migration, synapse formation (Dikranian et al., 2001; Udin & Grant, 1999), neurite growth (Udin & Grant 1999), spine formation (Ultanir et al., 2007) and neuronal plasticity (Fagiolini et al., 2003; Drian et al., 2001; Iwasato et al., 2000; du Bois & Huang 2007). The expression of neural cell adhesion molecules is partly controlled by NMDAR activation. The pharmacological blockade of NMDARs in neonatal rats causes a loss and disruption of synapses with profound abnormalities of brain structure and behaviour in adulthood (Dikranian et al., 2001). NMDAR blockade also produces a substantial loss of neurons partly resulting from the regulation by NMDARs of the p53 tumour suppressor gene. A similar loss of NMDAR function during development could contribute to the programmed elimination of neurons which shapes the early nervous system.

The major route for the metabolism of tryptophan is the oxidative pathway via kynurenine (Stone & Darlington, 2002). In most other tissues, including blood monocytes,

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macrophages and their descendant microglia, the first enzyme is indoleamine-2,3-dioxygenase [IDO], which is potently induced by inflammatory mediators such as IFN-γ, IL-1β and TNF-α which mediate the effects of lipopolysaccharides and poly(I:C). Since the pathway includes quinolinic acid as an agonist at NMDARs (Stone & Perkins 1981) and kynurenic acid as an antagonist at all ionotropic glutamate receptors, though with greatest potency at NMDARs (Perkins & Stone 1982), it represents a major link between activation of the immune system and the modulation of glutamate (especially NMDA) receptors. In addition, the kynurenine pathway is present partly in neurons and partly in glia (Guidetti et al., 2007; Schwarcz & Pellicciari, 2002; Guillemin et al., 2001, 2005). Since glial cells present in the early stages of CNS development regulate the appearance and function of NMDARs, the location of the pathway is consistent with the possibility that the activation of NMDARs is determined partly by quinolinic acid balanced by the glutamate receptor antagonist kynurenic acid. Indeed, it has been proposed that tissue formation and polarity may be determined largely by a gradient for a signal antagonist, rather than an agonist (Gurdon & Bourillot, 2001), which would fit well with the concept of a major developmental role for kynurenic acid. With its selectivity for NMDARs, kynurenic acid would also alter the balance of activation of the various subpopulations of glutamate receptors.

The kynurenine pathway is present during embryonic development (Beal et al., 1992; Saito et al., 1993; Walker et al., 1999), as reflected in the expression of one or more enzymes or the presence of key components such as kynurenine, kynurenic acid or quinolinic acid. The ability of prenatal inhibition of the kynurenine pathway to produce significant changes in synaptic properties in the adult offspring, as shown in this report, strongly suggests that the pathway normally plays a significant role in cerebral development. There are several ways in which this could occur, the most likely being changes in cerebral concentration of kynurenic acid which may vary during pregnancy in response to external factors such as infection and stress. Indeed any immune challenge to

the mother or neonate which results, directly or indirectly, in the activation of central glia or peripheral macrophages, or changes in the levels of cytokines or kynurenines in the foetal or neonatal CNS would alter the balance of quinolinic acid and kynurenic acid concentrations and could significantly affect neural development and plasticity. This proposal is consistent with evidence that genetic abnormalities of the kynurenine pathway are linked to disorders such as schizophrenia (Miller et al., 2006; Holtze et al., 2012).

Protein and mRNA expression

Analysis of mRNA levels using qRT-PCR failed to reveal any changes in gene expression. This strongly suggests that the various changes in synaptic function and protein expression have developed either from alterations in the translation of unchanged RNA into protein or from short-term changes in gene expression that may have been limited to the time during which Ro61-8048 was active in raising the levels of kynurenic acid. It should also be noted that temporal and quantitative differences are frequently encountered in comparisons of mRNA and protein expression, resulting in poor correlations between these parameters (English et al., 2011)

The investigation of protein expression was performed to identify changes that might result from kynurenine pathway inhibition. Many of the changes that had been recorded at P21 (Forrest et al., 2013) were no longer demonstrable in this study at P60, suggesting that those aspects of neuronal and synaptic development and function which were abnormal compared with control animals at P21 had largely returned to normal by P60. However, the existence of changes at P21 implies an effect of kynurenine pathway inhibition on both the extent of expression at this time and on the rate of appearance or disappearance of those proteins in the course of brain development. Clearly, either quantitative or temporal disturbances of protein expression are likely to lead to a degree of abnormal expression and synaptic function at P60. Since the P21 stage of postnatal

development in the rat brain is estimated to be equivalent to the early stage of brain development seen near the end of the second trimester of pregnancy in humans (Dobbing and Sands 1979) it is interesting to speculate that any factors affecting brain development at these times are likely to result in some degree of abnormal brain function.

Of the 9 target proteins whose expression was modified at P21, three of them are molecules that remain significantly changed at P60: the GluN2A subunit, doublecortin and sonic hedgehog. Of note is the finding that the difference in GluN2B expression seen at P21 was not apparent at P60 and that expression of GluN2A was significantly different but in the opposite direction to that at P21 – a decrease in expression compared with the earlier increase. Since the ratio between GluN2A and GluN2B subunits is known to change during the course of early brain development and to have a major influence on behavioural and electrophysiological outcomes by adulthood (Traynelis et al., 2010), the shifts we have reported in these two subunits may be among the more important factors in generating the functional synaptic changes described above. As noted earlier, the reduced LTP and GluN2A expression seen here are entirely compatible with the recently reported increase in expression of GluN2A induced by LTP (Baez et al., 2013).

Doublecortin

The increased expression of doublecortin at P60 – a change similar in magnitude and direction to that recorded at P21 - may provide an explanation for the electrophysiological changes reported here. Although the name 'doublecortin' was derived from the role of the protein in disorders of human neuronal migration in the neocortex which resulted in mental retardation and hyperexcitability (Gleeson et al., 1999; Nacher et al. 2001), the protein has also been linked with cellular lamination in the hippocampus (Corbo et al., 2002). It is a microtubule-associated protein which is expressed primarily in newly formed neurons (Couillard-Despres et al., 2005) and its deletion results in reduced neurogenesis and poor

recovery after stroke injury (Jin et al., 2010). Doublecortin is certainly active in neurogenesis during the earliest phases of brain development, with a progressive decline in its levels in the mature brain although it continues to be associated with new cell generation in the adult brain. The hippocampus, especially the subgranular zone of the dentate gyrus, exhibits high levels of doublecortin in the adult, probably related to the high levels of neurogenesis here. The significant increase in doublecortin levels, therefore, would imply an increased rate of new neurogenesis in the hippocampus following exposure to Ro61-8048 *in utero*.

While there were no overall changes in LTD, the more prolonged recovery from low frequency stimulation may be related to the association that has been reported between doublecortin expression and GABA-ergic function. The neuronal hyperexcitability which results from mutations or deletions of doublecortin results from a reduction of synaptic inhibition (Kerjan et al., 2009) which in turn is consistent with the co-localization of doublecortin with GABA, its synthetic enzyme glutamate decarboxylase, or parvalbumin, a marker for GABA-releasing neurons (Cai et al., 2009). Indeed, neurons expressing doublecortin may be primarily destined to become GABAergic interneurons (Wu et al., 2008; Xiong et al. 2008) and it has been proposed that doublecortin is required for the early migration of inhibitory interneurons in the cortex (Kappeler et al., 2006; Friocourt et al. 2007).

Sonic hedgehog

The only other protein to remain significantly changed at P60 was sonic hedgehog (Shh). This protein is involved in the early development of tissue polarization and the generation of morphogenetic and orientational gradients (Palma et al., 2005; Traiffort et al., 2010). Despite its importance in the earliest stages of CNS formation, it continues to exist in the adult brain, notably in regions such as the cerebellum (Vaillant & Monard

2009) and hippocampus (Traiffort et al., 1998; Charytoniuk et al., 2002; Dellovade et al., 2006; Ahn & Joyner, 2005) where neurogenesis continues in the adult. Shh may also modulate cell proliferation and the migration of progenitor cells (Traiffort et al., 2001; Charytoniuk et al., 2002; Palma et al., 2005; Sims et al., 2009; Dave et al., 2011).

In addition, Shh is thought to modulate the mutual attraction or repulsion between neuritic branches and potential sites of synaptic contact (Hor & Tang, 2010; Angot et al., 2008) and the reduction in Shh may interfere with appropriate contact formation during development. This may contribute to the functional differences observed in hippocampal transmission. In view of the evidence that Shh can promote the functional development of inhibitory neurons such as cerebellar granule cells (Spassky et al., 2008; Prajerova et al., 2010) the combination of these factors could be partly responsible for the observed decreased in paired-pulse inhibition.

Since doublecortin has been linked with neurogenesis, it is perhaps surprising that the levels of Shh protein decrease, while those of doublecortin increase, given the role of the former in neuronal orientation and contact formation. However, the roles attributed to doublecortin temporally precede those ascribed to Shh, leading to the possibility that there is increased production of new neurons which then mature and differentiate more slowly than in control animals.

Although several of the proteins examined were altered at P21 after exposure to Ro61-8048 *in utero*, the fact that no changes persisted into young adulthood at P60 suggests that the brain is able to adapt well to this particular interference. However, the protein and functional changes still persisting at P60 indicate that, despite any adaptations that have occurred between drug administration *in utero* and P21 or P60, the effects of kynurenine pathway inhibition *in utero* can clearly generate a sufficient disturbance of brain development that changes in three proteins with fundamental roles in the formation and maintenance of neuronal numbers, growth, connectivity and plasticity persist until early

adulthood. This conclusion raises the intriguing possibility that physiological or environmental influences on the kynurenine pathway *in utero* may contribute to brain function and behaviour in the postnatal years and adulthood. Since Shh plays a particularly prominent role in development of dopaminergic neuron projections (Wang et al., 1995; Hynes et al., 1995; Tang et al., 2010; Wu et al., 2012; Parish et al., 2008), its loss may be particularly relevant to the emergence of parkinsonian symptoms in adulthood.

Behaviour

The changes in neurodevelopmental proteins and synaptic physiology might be expected to lead to alterations in behaviour. The administration of kynurenine to adult or perinatal rats (Erhardt et al., 2004; Nilsson et al., 2006; Pocivavsek et al., 2012) or the depletion of kynurenic acid levels by deleting kynurenine aminotransferase (Potter et al., 2010) have demonstrated effects on learning in complex tasks such as pre-pulse inhibition (Erhardt et al., 2004; Nilsson et al., 2006), a water maze (Chess et al., 2007), stimulus discrimination (Alexander et al., 2012; Pocivavsek et al., 2012) or attentional set shifting (Alexander et al., 2013; Pocivavsek et al., 2012). In the present study behavioural testing was limited to a classical step-down avoidance task which can simplify the interpretation of drug effects on registration, consolidation and recall, but no significant differences were noted in the offspring of animals treated with Ro61-8048 compared with saline controls.

Differences between the results from these various studies may be caused by differences in the neurochemical effects of Ro61-8048 and kynurenine administration, the timing of prenatal treatment (three injections of Ro61-8048 during pregnancy in this study compared with kynurenine administration to adults or continual intake in the diet pre- and post-natally), or the nature of the behavioural tests. The simple step-down avoidance procedure may not be appropriate to detect cognitive changes that are only revealed in

more complex tasks (Chess et al., 2007; Pocivavsek et al., 2012; Alexander et al., 2012, 2013), or which require testing of aspects of behaviour more subtle than a simple learning task. It has also been noted previously that some, but not all aspects of cognitive function are influenced by elevated levels of kynurenic acid (Chess and Bucci, 2006; Chess et al., 2009). In addition, it has been reported that simpler learning tasks such as step-down avoidance task can be achieved by neural pathways involving or excluding the hippocampus, so that even major hippocampal damage can be circumvented by alternative cerebral pathways (Martel et al., 2010). This would certainly account for the failure of the subtle hippocampal changes described here to alter step-down avoidance.

Additional consequences of KMO inhibition

In addition to the major increase in kynurenic acid concentrations, the inhibition of KMO may also have effects on neuronal excitability and function by reducing the levels of other compounds normally generated as secondary products of kynurenine oxidation (Stone & Darlington 2002, 2013; Schwarcz et al., 2012; Stone et al., 2013). The immediate product of KMO, 3-hydroxykynurenine (3HK) is an effective neurotoxin (Okuda et al., 1998) and is converted by kynureninase to 3-hydroxy-anthranilic acid (3HAA) which, in addition to direct actions on T cell viability (Munn 2011), can auto-oxidise to the dimeric cinnabarinic acid (Dykens et al., 1987), a compound which is at least 10-fold more active as an inducer of T cell death (Hiramatsu et al., 2008). This compound also activates the metabotropic glutamate receptor mGluR4 (Fazio et al., 2012) which, as a member of the Group III metabotropic receptor family, has a range of actions in the CNS including modulation of anxiety, depression (Wieronska et al. 2010) and basal ganglia function (Niswender et al., 2008; Broadstock et al., 2012). Another potentially important product is xanthurenic acid which has several characteristics of a neurotransmitter candidate (Gobaille et al. 2008), activates group II metabotropic glutamate receptors and can modify subcortical

neurotransmission (Copeland et al., 2013). It may also activate cationic channels that enhance neuronal excitability (Taleb et al., 2012) and may modulate excitability indirectly via inhibition of vesicular glutamate transporters (Neale 2013).

Summary

Overall, the evidence from this study suggests that there is a basal, constitutive level of activity in the kynurenine pathway which is normally active in the embryonic, developing CNS. The pathway can influence the composition and function of NMDA receptors and the expression of proteins critical to normal brain development and function, some of which can persist into adulthood. These changes result in abnormal neurotransmission and plasticity which could cause disease or predispose individuals to the development of CNS disorders. The changes seen at P60 are remarkable in several respects. Firstly, many of the changes in protein expression seen at P21 are no longer present at P60, suggesting that the influence of modifying kynurenine function prenatally produced temporary, not permanent, alterations. The early changes, however, may well have caused or contributed to the changes noted here.

Secondly, the functional changes in synaptic interactions and plasticity are different at P60 from those seen at P21, indicating that the processes of network development and possible remodelling have been significantly modified by the earlier effects of Ro61-8048.

Thirdly, since the kynurenine pathway (mainly IDO and KMO) is activated by interferons and pro-inflammatory cytokines (Alberati-Giani et al., 1996), the ability of the pathway to regulate brain development carries the implication that it could be responsible for some of the recognised influence of infection during pregnancy on brain development of the embryo (Brown 2006, 2011; Meyer and Feldon 2010; Hornig et al., 1999). There are also some compounds in the diet, such as the brassinins found in cruciferous vegetables (cabbage, broccoli etc), which inhibit the kynurenine pathway (Banerjee et al., 2008), mimicking the effects of Ro61-8048. According to our results the changes in the levels of

kynurenic acid and other kynurenine metabolites which they could produce might affect brain development. There are also simple metabolites such as oxoglutarate and pyruvate (Shin et al., 1982) as well as vitamins such as pyridoxal (Breton et al. 2000) which can activate or inhibit KMO directly depending on concentration and which might have pathological relevance.

Finally, two of the proteins that are fundamental players in the early generation, maturation and organisation of cells remain significantly altered at P60 in the same direction as found at P21 – doublecortin levels are increased and sonic hedgehog levels are decreased at both time points. Together with the persisting increase in GluN2A expression it seems probable that these proteins might represent key links between the kynurenine pathway and brain development and future work should be directed at exploring the sites and mechanisms of this relationship.

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Figure legends

Figure 1

Hippocampal function at P60 after prenatal exposure to Ro61-8048.

The increase in size of (A) field excitatory postsynaptic potentials (EPSPs) and (B) population spikes (PS) as a function of stimulus current in the hippocampal CA1 region of adult (P60) rats exposed prenatally to Ro61-8048. The symbols indicate mean $\pm$ s.e.mean (n = 12). There were no significant differences between any of the pairs of data points. Paired-pulse data are shown for the ratio of fEPSPs recorded in the stratum radiatum (C). The sample recordings alongside the graph illustrate the digital subtraction performed for the recordings made at an interpulse interval of 10ms, with (a) being a normal single fEPSP, (b) two responses at the 10ms interval, and (c) the second response alone generated by the subtraction of (a) from (b). Record (d) shows the directly superimposed traces (a) and (c) while (d) illustrates the superimposed traces adjusted to the same stimulus point. Paired pulse data for the ratio of PS recorded in stratum pyramidale are shown in (D). The sample traces alongside illustrate (a) a single PS while other traces illustrate pairs of PS at interpulse intervals of (b) 50ms (c) 20ms and (d) 10ms.

The change in PS ratio at different rates of paired-pulse presentation from 0.05 to 1Hz is shown in (E). The sample traces show individual records of paired PS at presentation rates of (a) 0.05Hz and (b) 1Hz.

Symbols in the graphs indicate mean $\pm$ s.e.mean (n = 12).

** P < 0.01; ***P < 0.001 using the Bonferroni test for multiple comparisons following ANOVA..

Figure 2.

Adult hippocampal plasticity after prenatal exposure to Ro61-8048.

Panel (**A**) illustrates long-term potentiation in the two groups of offspring following theta-burst stimulation at the arrow. The inset records illustrate a control fEPSP immediately before stimulation and the potentiated response 40min later in a slice from a control animal (**B**) or an animal exposed in utero to Ro61-8048 (**C**). The symbols indicate mean ± s.e.mean (n = 6). Calibrations 0.5mV and 10ms.

*** $P < 0.001$ for the final 5 minutes of recording (ANOVA).

Panel (**D**) illustrates long-term depression in the two groups of offspring induced by two periods of low frequency stimulation indicated by the bars on the abscissa at stim1 and stim2 (see text). The inset records illustrate a control fEPSP immediately before stim1 and the depressed responses 20min after stim1 and 20 min after stim2 in a slice from a control animal (**E**) or an animal exposed in utero to Ro61-8048 (**F**).

The symbols indicate mean ± s.e.mean (n = 6). Calibrations 1mV, 10ms for the control records and 0.5mV, 10ms for drug-exposed slices. There were no significant differences between levels of LTD in the control and treated slices over the last 5min of recording.

Figure 3.

Sensitivity to adenosine of adult hippocampal slices after prenatal exposure to Ro61-8048.

(A) Graph summarising changes in fEPSP amplitude in response to the application of adenosine at concentrations of 10μM and 30μM. Adenosine was superfused for 10min indicated by the solid bar. Symbols represent responses recorded in control slices superfused with 10μM (filled squares,■), or 30μM (filled circles,●), and slices from animals exposed prenatally to Ro61-8048 superfused with 10μM (open diamonds, ◊) or 30μM

(open triangles, Δ) adenosine. (B) and (C) show individual sample records of fEPSPs from control (B) or Ro61-8048 exposed animals (C), recorded before and after recovery from the application of adenosine at 30µM, and at the peak of the response to adenosine, illustrating complete recovery after the adenosine application. (D) summarises the pooled data on the degree of fEPSP depression produced by adenosine at 10µM and 30µM, illustrating the absence of any difference between slices from control and Ro61-8048 treated animals.

Calibrations 1mV, 10ms.

Figure 4

Analysis of mRNA by qRT-PCR.

Effect of prenatal administration of Ro61-8048 on mRNA expression in the hippocampus of offspring at P60. qRT-PCR analysis of the expression of transcripts (*Grin2a, Grin2b, Dcx, Shh*) in relation to 3 reference genes *Actb, Gapdh and Rn18s. Grin2a* (A, E, J), *Grin2b* (B, F, K), *Dcx* (C, G, L) and *Shh* (D, H, M). Results are shown as mean ± s.e.m. of $2^{-\Delta Ct}$ (n = 9 animals per group).

Figure 5

Expression of proteins in the hippocampus of control and Ro61-8048-treated animals.

The panels summarise the expression of several of the proteins examined in this study using Western blots. Data are shown for the optical density of blots relative to actin for (A) GluN2A, (B) GluN2B and (C) GluN1 subunits, (D) PSD-95, (E) RhoA, (F) EphA4, (G) doublecortin (DCX) and (H) sonic hedgehog (Shh).

* $p < 0.05$; ** $p < 0.01$ (t test, n = 6 for Ro61-8048, n = 4 for controls).

Figure 6

Open-field exploration task.

Animals were exposed twice to an open field arena on two consecutive days. The bar diagrams summarize (A) the number of times the animals cross into new sectors of the open field arena (OF) and (B) the number of rearings for control and treated rats, during the 5 min periods they were exposed to the arena. Results are shown from the first session (OF1) and from the second session (OF2) performed 27-28h later.

The bars represent median with interquartile ranges.

* significant difference between control and treated animal ($p < 0.05$, Wilcoxon signed rank test after Kruskal–Wallis ANOVA for non-parametric samples).

All animals: control n = 38; treated n = 53.

There were no significant differences between control and treated groups in crossings and rearings during the first (OF1, P>0.05) or second open field exposure (OF2; p>0.05, Mann-Whitney test).

Figure 7

Step-down inhibitory avoidance in control and treated animals.

The bar diagrams show the latencies to step-down from an isolated platform to the electrified grid, in an inhibitory avoidance paradigm. Control and treated rats were trained (TR) and tested (TEST) after 20 h. The bars represent median with interquartile ranges. TR: training session; TEST: test session; DIFF: differences between test and training sessions).

* significant difference between TR and TEST latencies ($p < 0.05$, Wilcoxon signed rank test after Kruskal–Wallis ANOVA for non-parametric samples). (A) All animals were 60 day old adults treated prenatally with Ro61-8048: control n=34; treated n=50. Analysis was also performed for males and females separately to exclude any gender effects: (B) male rats: control n=20; treated n=19; (C) female rats: control, n=14; treated, n=31.

There were no significant differences between groups in latency differences ($p > 0.05$, Mann-Whitney test).
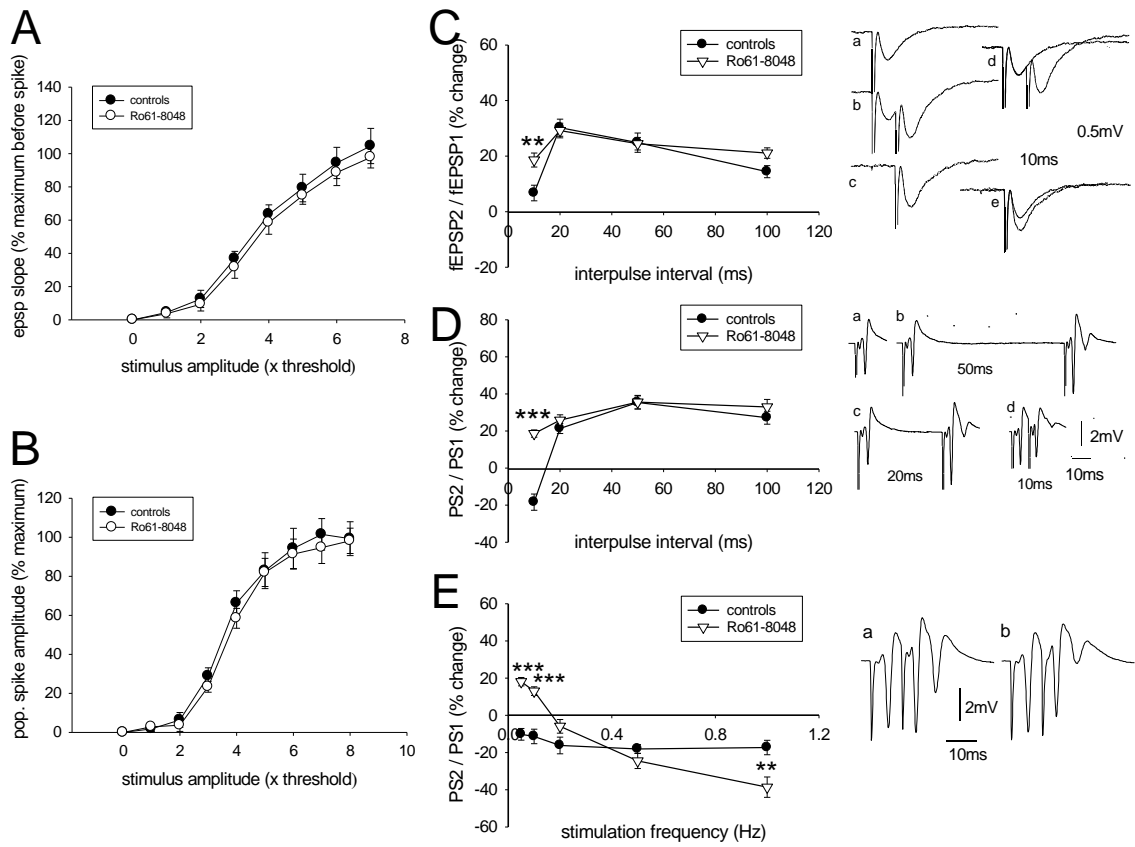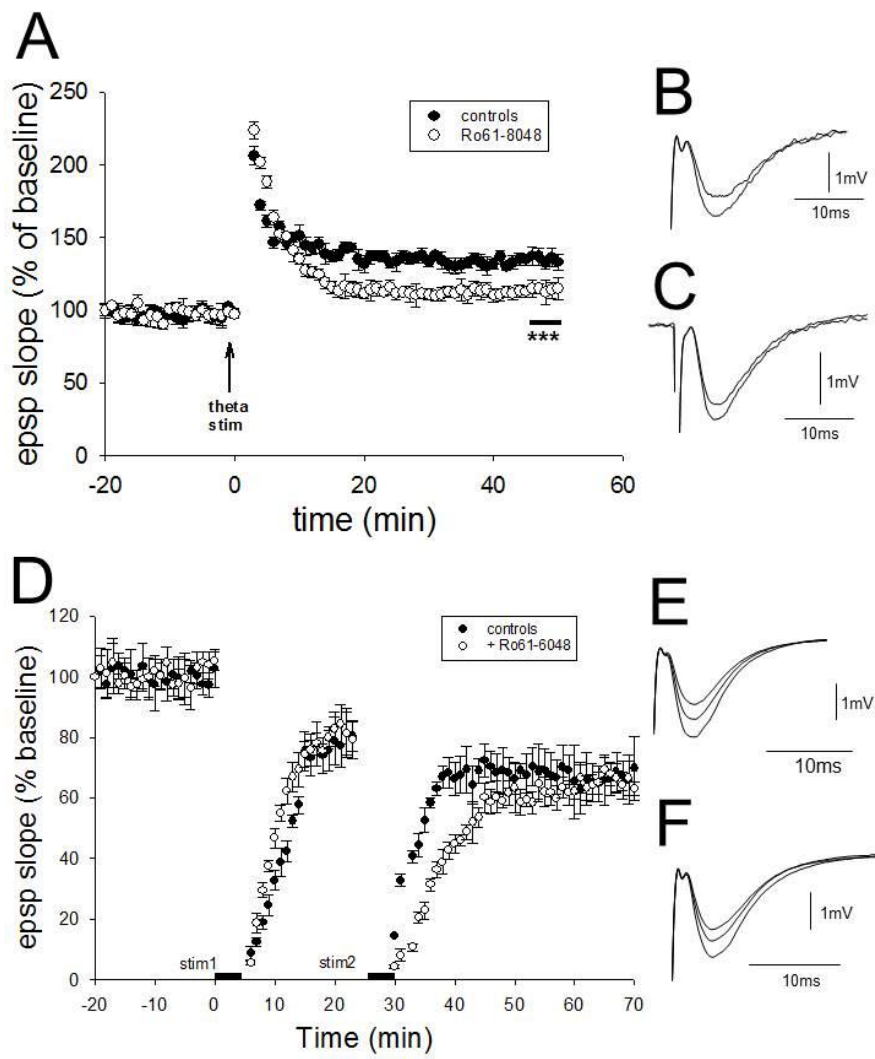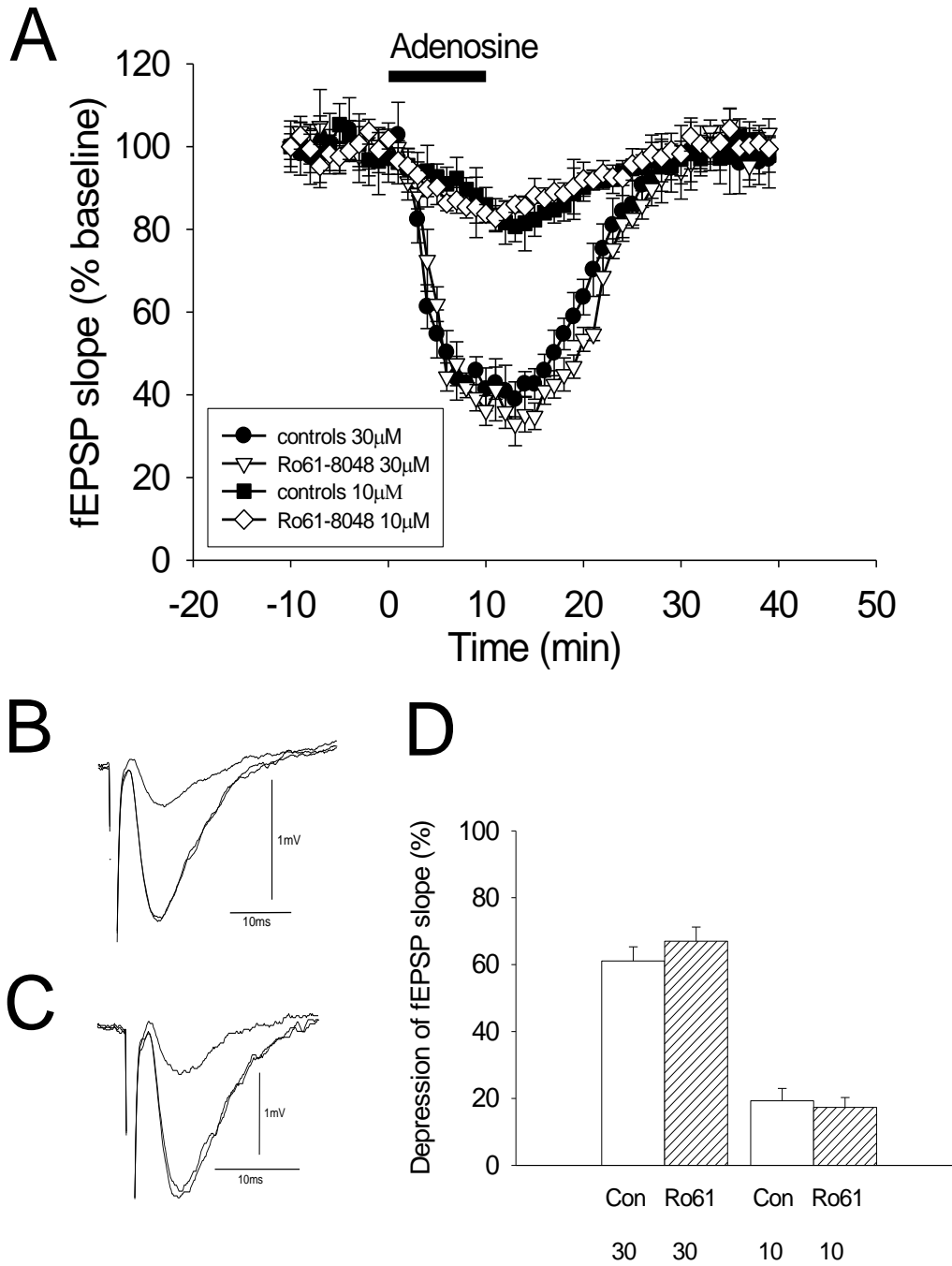
Figure 1

Figure 2

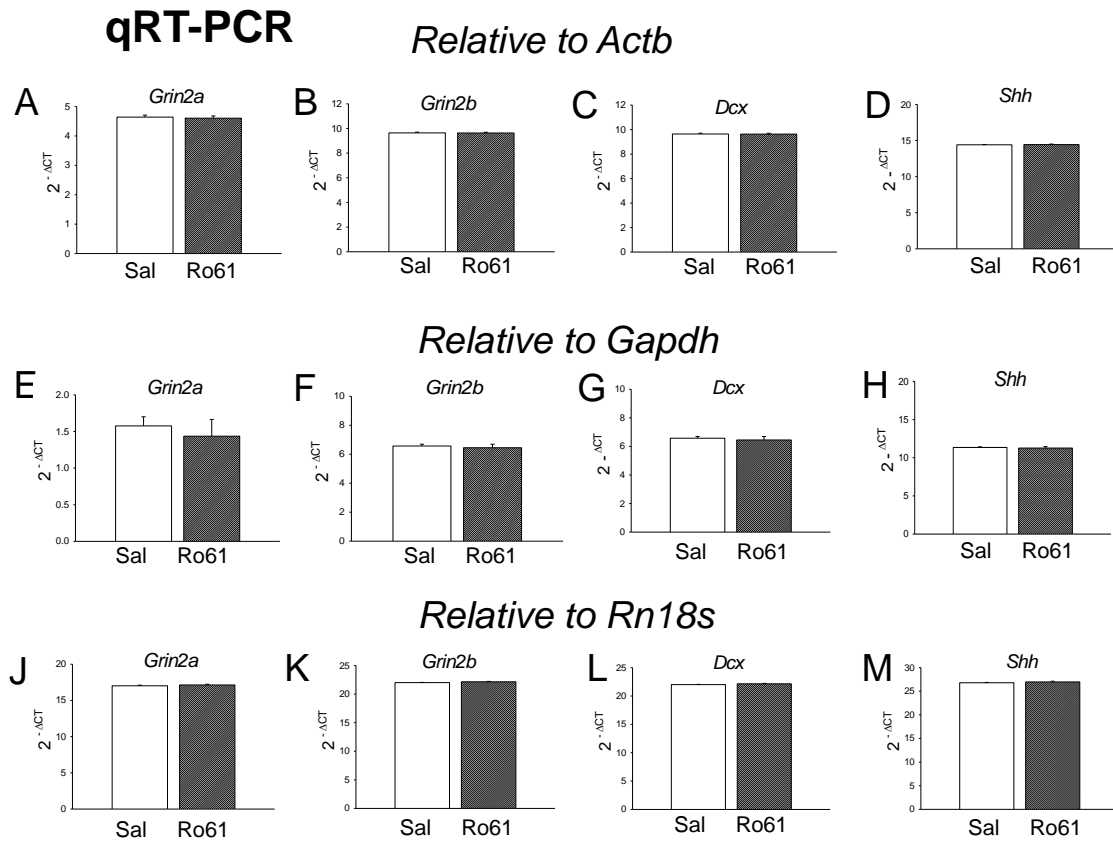Figure 3

Figure 4

qRT-PCR

Relative to Actb

Relative to Gapdh

Relative to Rn18s

Figure 5

Figure 6

Figure 7

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