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CYTOARCHITECTURAL DISRUPTION OF THE SUPERIOR COLLICULUS AND AN ENLARGED ACOUSTIC STARTLE RESPONSE IN THE *Tuba1a* MUTANT MOUSE

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Abstract—The *Jenna* mutant mouse harbours an S140G mutation in Tuba1a that impairs tubulin heterodimer formation resulting in defective neuronal migration during development. The consequence of decreased neuronal motility is a fractured pyramidal cell layer in the hippocampus and wave-like perturbations in the cerebral cortex. Here, we extend our characterisation of this mouse investigating the laminar architecture of the superior colliculus (SC). Our results reveal that the structure of the SC in mutant animals is intact; however, it is significantly thinner with an apparent fusion of the intermediate grey and white layers. Birthdate labelling at E12.5 and E13.5 showed that the S140G mutation impairs the radial migration of neurons in the SC. A quantitative assessment of neuronal number in adulthood reveals a massive reduction in postmitotic neurons in mutant animals, which we attribute to increased apoptotic cell death. Consistent with the role of the SC in modulating sensorimotor gating, and the circuitry that modulates this behaviour, we find that *Jenna* mutants exhibit an exaggerated acoustic startle response. Our results highlight the importance of *Tuba1a* for correct neuronal migration and implicate postnatal apoptotic cell death in the pathophysiological mechanisms underlying the tubulinopathies.

Key words: tubulin lissencephaly, superior colliculus, acoustic startle response.

The *Jenna* (*Jna*/+) mouse, generated from an *N*-ethyl-*N*-nitrosourea mutagenesis screen, has a dominantly inherited mutation in exon four of the α-tubulin gene, *Tuba1a* (Keays et al., 2007). This mutation, an S140G substitution, impairs tubulin heterodimer formation, which results in defects in neuronal migration during development. Consequently, mutant animals have a fractured pyramidal cell layer in the hippocampus, and laminar abnormalities in the cerebral cortex, predominantly in layers III and IV. Human studies have found that this mouse is a model for lissencephaly, a disorder characterised by simplified gyration of the cortex, mental retardation and epilepsy (Guerrini and Marini, 2006). Mutations in a number of genes cause lissencephaly, including DCX, LIS1, VLDLR and the *reelin* gene (des Portes et al., 1998; Gleeson et al., 1998; Hong et al., 2000; Boycott et al., 2005). In the later case, homozygous mutations in reelin result in gyral simplification, a thickened cortex and cerebellar hypoplasia (Hong et al., 2000).

Reelin, a large extracellular protein, was first implicated in neuronal migration after the identification of deletions in the *reeler* mouse, which is noted for its inverted cortex and disorganised hippocampus and cerebellum (D'Arcangelo et al., 1995). More recently, the catalogue of neuroanatomical abnormalities in the *reeler* mouse has been extended to the superior colliculus (SC). In vertebrates, the SC consists of seven layers that are both anatomically and functionally organised. The superficial SC consists of the three uppermost layers: the zonal (Zn), superficial grey (SuG) and the optic layer (Op), and the deep SC contains four layers: the intermediate grey (InG), intermediate white (InW), deep grey (DpG) and deep white (DpW). In the reeler mouse, it has been reported that the superficial layers of this structure are cytoarchitectually and myeloarchitectually disorganised (Baba et al., 2007). Similarly, disruption of the laminar patterning in the SC has been observed in the *reelin*-deficient shaking rat Kawasaki (Sakakibara et al., 2003).

Given that both mutations in reelin and TUBA1A cause similar phenotypes in humans, in this article, we set out to investigate the cytoarchitecture of the SC in the *Jna*/+ mice. Using histological tools, we found that the laminar structure of the SC in mutant animals was intact; however, it was significantly thinner with an apparent fusion of the InG and InW. Using birthdate labelling, we showed that the neuronal migration defect that is observable in the cortex and the hippocampus of affected animals is also apparent during the formation of the SC. Additionally, an elevated rate of cell death leads to a significant loss of neurons in the SC of the *Jna*/+ mouse between postnatal day 21 (P21) and 12 weeks of age, with the majority of the loss occurring in the deep layers. Consistent with the role of the SC in modulating sensorimotor gating, we observed an

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Abbreviations: BrdU, 5-bromo-2-deoxyuridine; DAPI, 4',6-diamidino-2-phenylindole; DpG, deep grey layer; DpW, deep white layer; InG, intermediate grey layer; InW, intermediate white layer; *Jenna*, *Jna*/+; Op, optic layer; PAG, periaqueductal grey; SC, superior colliculus; SuG, superficial grey layer; Tpd52L1, tumour protein D52-like-1; Zn, zonal layer.

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exaggeration of the acoustic startle response in mutant animals.

EXPERIMENTAL PROCEDURES

Animals

Mice were maintained on a C3H/HeH (Harlan, UK) background and housed on a 12:12 light:dark cycle at a temperature of 22±1 °C and humidity of 60%–70%. Males and females were separated at weaning (P21) and housed separately in groups of five where possible. The genotype of animals was determined by polymerase chain reaction analysis, as previously described (Keays et al., 2007), and only littermates were selected for experiments. Cages were environmentally enriched with cardboard tubing, and mice were permitted *ad libitum* access to food. Experiments were performed in accordance with the UK Animals (Scientific Procedures) Act 1986.

5-bromo-2-deoxyuridine (BrdU) labelling

For birthdate labelling experiments, pregnant C3H females were injected with BrdU (50 μg/g of body weight), 12.5 and 13.5 days after copulation. All resultant P0 pups were killed and genotyped. P0 brains were extracted and drop-fixed in 4% paraformaldehyde for 4 h before being placed in sucrose overnight. Brains were then embedded in OCT and sectioned at 14 μm using a cryostat, then mounted onto electrostatic slides and stored at −20 °C. Prior to staining, antigen retrieval was performed in citrate buffer at 90 °C (0.01 M, pH 6). To quench peroxidase activity, slides were placed in 3% H_2O_2 solution for 10 min, followed by three washes in PBS. Sections were then digested in trypsin (0.0125%) for 10 min at 37 °C, washed three times in PBS, followed by a 20-min incubation in 2 N HCl at 37 °C. After washing, slides were incubated in a 0.3% Triton/PBS with 2% rabbit serum (Rat Elite ABC Kit, Vector Labs, Peterborough, UK) and 1:100 rat anti-human BrdU antibody (Accurate Chemical & Scientific, Westbury, NY, USA) in a humidity chamber overnight. Slides were then washed and stained as directed in the Elite ABC Kit, using DAB permanent staining.

Gallyas staining

Twelve-week-old *Jna*/+ mice and wild-type (WT) littermates were perfused with 0.9% saline and 10% formalin solution. Brains were extracted and left in 10% formalin solution for 2 weeks. Subsequently, brains were cryoprotected in 30% sucrose/formalin and sectioned on a freezing microtome (25 μm) before storing in 5% formalin solution at 4 °C for a further 2 weeks. Sections were matched, mounted on electrostatically charged slides and stained using a Gallyas silver staining method based on study by Pistorio et al. (2006).

In situ hybridisation

A 429-base pair probe targeting the tumour protein D52-like-1 (Tpd52L1) gene was generated by PCR using the following primers: Tpd521l_F GAAAGTAGGTGGGACAAACCAC and Tpd521l_R GGAGACCAAGTCAAAACCAAAG and cloned into a pCR2.1-TOPO vector (Invitrogen, CA, USA). Digoxigenin-labelled riboprobes were then synthesized using appropriate RNA polymerases (Roche Applied Science, Burgess Hill, UK), and hybridisation was performed, as previously described (Isaacs et al., 2003). All hybridisations were performed on 14-μm brain sections prepared from *Jna*/+ mice and WT littermates aged 12 weeks.

Juvenile and adult immunohistochemistry

Cohorts of P21 and 12-week-old *Jna*/+ and WT littermate mice (n=4) were perfused with 0.9% NaCl and 4% paraformaldehyde, and brains were postfixed for 6 h, followed by dehydration in 30% sucrose solution. Sections (40 μm) were prepared on a freezing microtome and stored in antifreeze solution at −20 °C. For each P21 and 12 week brain, three matched SC sections were selected for staining. Sections were incubated with the primary antibody overnight in 0.3% Triton/PBS with 2% of the appropriate serum at the following concentrations: NeuN (1:500) (Millipore, Billerica, MA, USA); Calbindin (1:500) (Millipore, Billerica, MA, USA); Er81 (1:4000). After three washes in PBS (5 min each), sections were incubated with a biotinylated secondary antibody (1:500) in 0.3% Triton/PBS for 2 h. After a series of washes in PBS, sections were incubated for 1 h with fluorescein-conjugated streptavidin (1:500) (Vector Labs, Peterborough, UK). Sections were mounted on electrostatic slides andcoverslipped with 4',6-diamidino-2-phenylindole (DAPI) containing mounting media (Vector Labs, Peterborough, UK).

Apoptosis study

Seven-week-old mice *Jna*/+ and WT littermate mice were perfused and sectioned in the aforementioned manner (n=5). Every 8th section from the beginning of the SC through to the start of the inferior colliculus was mounted on electrostatic slides. Prior to staining, slides underwent antigen retrieval, peroxidase quenching and PBS washing. Sections were incubated with the caspase 3 primary antibody (1:400) (Cell Signaling Technology, Boston, MA, USA) overnight in 0.3% Triton/PBS with 2% goat serum. Slides were then washed and stained, as directed in the Elite ABC Kit, using DAB permanent staining. Total cell counts were then obtained by multiplying the number of positive cells observed by a factor of 8.

Quantification and statistics

The SC was analysed through the creation of 10 zonal analysis boxes in which cells could be counted. Boxes were drawn on SC images using ImageJ freeware, were positioned 100 μm from the midline and were a consistent 250 μm in width. The boxes were of equal height within an image, with the height of each box being a 10th of the distance between the surface of the Zn to a depth in the periaqueductal grey (PAG) level with the cerebellar aqueduct. Once the analysis boxes were formatted, labelled cells within each box were counted manually. The percentage of neurons within each box was calculated from the number of NeuN staining cells as a proportion of DAPI cells. Statistics were performed in GraphPad Prism. A 1-way ANOVA with a Bonferroni correction for multiple comparisons was used for the analysis of behavioural measurements and for NeuN, BrdU and DAPI cell density zonal measurements.

Behavioural testing

The acoustic startle response was measured using a commercially available behavioural testing system (San Diego Instruments, San Diego, CA, USA). This apparatus consisted of a sound-proof box with a 6-cm speaker to deliver the acoustic stimuli, coupled to an accelerometer to monitor the animal's movement. The behavioural testing was performed in a separate room, and the cages were moved into the room at least 2 h before the start of the experiment. Prior to testing, each mouse was placed inside the startle apparatus and allowed to acclimatise for 5 min. To test the acoustic startle response, acoustic stimuli were presented as 40-ms impulses of white noise with four different intensities (90, 100, 110 and 120 dB) for a total of 20 times, each in a pseudorandom order and spaced at random intervals between 10 and 20 s. Responses were expressed in arbitrary units and averaged for each type of trial.