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INCREASED SUSCEPTIBILITY TO KAINIC ACID–INDUCED SEIZURES IN *Engrailed-2* KNOCKOUT MICE

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Abstract—The *En2* gene, coding for the homeobox-containing transcription factor Engrailed-2 (EN2), has been associated to autism spectrum disorder (ASD). Due to neuroanatomical and behavioral abnormalities, which partly resemble those observed in ASD patients, *En2* knockout (*En2*$^{-/-}$) mice have been proposed as a model for ASD. In the mouse embryo, *En2* is involved in the specification of midbrain/hindbrain regions, being predominantly expressed in the developing cerebellum and ventral midbrain, and its expression is maintained in these structures until adulthood. Here we show that in the adult mouse brain, *En2* mRNA is expressed also in the hippocampus and cerebral cortex. Hippocampal *En2* mRNA content decreased after seizures induced by kainic acid (KA). This suggests that *En2* might also influence the functioning of forebrain areas during adulthood and in response to seizures. Indeed, a reduced expression of parvalbumin and somatostatin was detected in the hippocampus of *En2*$^{-/-}$ mice as compared to wild-type (WT) mice, indicating an altered GABAergic innervation of limbic circuits in *En2*$^{-/-}$ mice. In keeping with these results, *En2*$^{-/-}$ mice displayed an increased susceptibility to KA-induced seizures. KA (20 mg/kg) determined more severe and prolonged generalized seizures in *En2*$^{-/-}$ mice, when compared to WT animals. Seizures were accompanied by a widespread c-*fos* and c-*jun* mRNA induction in the brain of *En2*$^{-/-}$ but not WT mice. Long-term histopathological changes (CA1 cell loss, upregulation of neuropeptide Y) also occurred in the hippocampus of KA-treated *En2*$^{-/-}$ but not WT mice. These findings suggest that *En2*$^{-/-}$ mice might be used as a novel tool to study the link between epilepsy and ASD. **© 2009 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: autism spectrum disorder, epilepsy, homeobox, hippocampus, glutamate.

The homeobox-containing transcription factor Engrailed-2 (EN2) is crucially involved in the regionalization, patterning and neuronal differentiation of the midbrain and hindbrain, which represent the regions of the CNS in which its expression has been detected. Indeed, *En2* is predominantly expressed in the developing cerebellum and ventral midbrain, starting at the neural plate stage (E8.5) and continuing throughout embryonic and postnatal development (for reviews see Joyner, 1996; Herrup et al., 2005; Hidalgo-Sánchez et al., 2005; Gherbassi and Simon, 2006). Initial studies performed on *En2* knockout (*En2*$^{-/-}$) mice revealed a phenotype consistent with this restricted expression pattern. *En2*$^{-/-}$ mice displayed cerebellar hypoplasia, a reduced number of Purkinje cells and a subtle but reproducible defect in the anteroposterior pattern of cerebellar foliation (Joyner et al., 1991; Millen et al., 1994, 1995; Kuemerle et al., 1997). More recently, deficits in social behaviors were detected in *En2*$^{-/-}$ mice, including decreased play, reduced social sniffing and allogrooming, and reduced aggressiveness (Cheh et al., 2006). Deficits in spatial learning and memory tasks (Morris water maze and object recognition test), as well as in specific motor tasks (rotarod) were also reported in *En2*$^{-/-}$ mice (Cheh et al., 2006). These studies suggest that *En2* might be expressed also in more anterior brain areas during adulthood.

Due to their complex neurodevelopmental, neuroanatomical and behavioral phenotype, *En2*$^{-/-}$ mice have been proposed as a novel model for autism spectrum disorder (ASD). Indeed, abnormalities observed in *En2*$^{-/-}$ mice resemble—at least in part—some of those that have been reported in ASD patients, such as hypoplasia of cerebellar vermal lobules (Courchesne et al., 1988). Many studies also reported a significant reduction in Purkinje cell number, cerebellar nuclei and inferior olive in ASD individuals (Williams et al., 1980; Bauman and Kemper, 1985; Bauman, 1991; reviewed in DiCicco-Bloom et al., 2006), as also observed in *En2*$^{-/-}$ mice (Kuemerle et al., 1997). More recently, altered anatomical structure of the amygdala has also been reported in *En2*$^{-/-}$ mice (Kuemerle et al., 2007). It is interesting to notice that in ASD patients, significant neuropathological alterations have been described in several telencephalic structures, including the amygdala, hippocampus and other limbic areas (reviewed in Palmen et al., 2004; Bauman and Kemper, 2005; DiCicco-Bloom et al., 2006). Finally, the human *En2* gene maps to 7q36, a chromosomal region that has been linked

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Abbreviations: ASD, autism spectrum disorder; *En2*, engrailed-2; IEGs, immediate early genes; KA, kainic acid; NPY, neuropeptide Y; RT-PCR, reverse transcription–polymerase chain reaction; WT, wild-type.

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to ASD, and two single-nucleotide polymorphisms (SNPs) in the *En2* gene have been associated to ASD (Gharani et al., 2004; Benayed et al., 2005; Brune et al., 2008).

Since a high prevalence of epilepsy has been described in ASD patients (Deonna and Roulet, 2006; Canitano, 2007), we studied seizure susceptibility in $En2^{-/-}$ mutant mice. Seizures were evoked by systemic administration of the glutamate receptor agonist kainic acid (KA), a widely used model to study the behavioral and neuropathological consequences of seizures in rodents (Lothman and Collins, 1981; Sperk, 1994; Schauwecker and Steward, 1997). The effects of KA administration in wild-type (WT) and $En2^{-/-}$ mice were evaluated by means of behavioral and histological analyses. Here we show that $En2^{-/-}$ mice display increased susceptibility to KA-induced seizures and long-term histopathology.

EXPERIMENTAL PROCEDURES

Animals

The generation of $En2^{-/-}$ mice was previously described (Joyner et al., 1991). The original *En2* mutants (mixed 129 Sv×Swiss–Webster genetic background) were crossed at least three times into a C57BL/6 background. $En2^{+/-}$ heterozygous mice of this background were obtained from the central animal facility of the University of Heidelberg, and used as founder animals to establish our own colony at the University of Pisa. Because $En2^{-/-}$ mice are viable and fertile (Joyner et al., 1991), two separate $En2^{+/+}$ (WT) and $En2^{-/-}$ colonies of the same genetic background were established, according to previous studies (Cheh et al., 2006; Kuemerle et al., 2007). This was obtained as follows: from heterozygous mating ($En2^{+/-} \times En2^{+/-}$), WT and $En2^{-/-}$ mice were identified by PCR genotyping (Sgadò et al., 2006) and used to establish the two different colonies. The WT and $En2^{-/-}$ colonies were maintained by ($En2^{+/+} \times En2^{+/+}$) and ($En2^{-/-} \times En2^{-/-}$) mating, respectively. For this reason, WT and $En2^{-/-}$ mice used in this study were not littermates; however, age-matched adult (5 months old; weight=25–35 g) male mice were used in all experiments. Animals were housed in a 12-h light/dark cycle with food and water available *ad libitum*. Experiments were conducted in conformity with the European Communities Council Directive of November 24, 1986 (86/609/EEC). All experiments conformed to the Italian Ministry of Health guidelines on the ethical use of animals. Care was taken to minimize the number of animals used and their suffering.

Quantitative reverse transcription–polymerase chain reaction (RT-PCR)

Total RNAs were extracted by Trizol© reagent (Invitrogen, Milan, Italy) from the cerebral cortex, hippocampus, ventral midbrain and cerebellum of four adult WT mice and pooled. For KA experiments, RNAs were extracted and pooled from hippocampi of four adult WT mice, killed 3 h after i.p. administration of KA (KA; Ocean Produce International, Shelburne, NS, Canada) at the dose of 20 mg/kg KA-treated mice experienced generalized seizures within the first 2 h after KA administration (see also below for seizure rating scale). RNA extracted from the hippocampus of four adult WT mice treated with saline was used as a control. DNase-treated RNAs were purified and concentrated with Nucleospin RNA columns (Macherey-Nagel, Düren, Germany). cDNA for real-time PCR was synthesized from RNA (2 μg) using the reverse transcriptase Core kit (Eurogentec, Seraing, Belgium) according to the manufacturer's instructions. Quantitative PCR was performed using a Rotor-gene 2000 thermal cycler with real-time detection of fluorescence (Corbett Research, Sydney, Australia). PCR reactions were conducted in a volume of 25 μl using the Mesa Green qPCR kit (Eurogentec) according to manufacturer's instructions. Mouse mitochondrial ribosomal protein L41 (Mrpl41) was used as a standard for quantification. Primers (Sygma-Genosys, Milan, Italy) were as follows: *En2* forward 5′-agagagggcgcagttctttg-3′; *En2* reverse 5′-cgacacagacgcagacacac-3′ (GenBank accession no. NM_010134.3; expected fragment size: 151 bp); L41 forward 5′-GGTTCTCCCTTTCTCCCTTG-3′; L41 reverse 5′-GCACCCCGACTCTTAGTGAA-3′ (GenBank accession no. NM_001031808.2; expected fragment size: 179 bp). Each PCR cycle consisted of denaturation for 10 s at 94 °C, annealing for 20 s at 62 °C (60 °C for L41), and extension for 30 s at 72 °C. The fluorescence intensity of SYBR Green I was read and acquired at 72 °C after completion of the extension step of each cycle. PCR conditions for individual primer sets were optimized by varying template cDNA and magnesium ion concentration in order to obtain amplifications yielding a single product and melt curves with a single uniform peak. Quantification of individual transcripts was performed using the dComparative QuantitationT software supplied with Rotor-gene. *En2* and L41 mRNA concentrations in ventral midbrain, cerebral cortex and hippocampus were referred to those detected in the cerebellum (comparative quantitation). In KA experiments, *En2* and L41 mRNA concentrations in the hippocampus of KA-treated mice were referred to those detected in the hippocampus of saline-treated animals. Ratios of *En2* mRNA/L41 mRNA comparative concentrations were then calculated and plotted as the average of three different technical replicates obtained from each RNA pool.

Behavioral observation of KA-induced seizures

KA (Ocean Produce International, Shelburne, NS, Canada) was dissolved in saline and administered i.p. at 20 mg/kg body weight. Eight WT and 12 $En2^{-/-}$ mice were used. In all experiments, the experimenter was blind to the genotype of the animals. Seizure severity was determined according to Racine's scale (Racine, 1972): stage 0: normal behavior; stage 1: immobility; stage 2: forelimb and/or tail extension, rigid posture; stage 3: repetitive movements, head bobbing; stage 4: forelimb clonus with rearing and falling (limbic motor seizure); stage 5: continuous rearing and falling; stage 6: severe whole body convulsions (tonic–clonic seizures); stage 7: death. For each animal, the rating scale value was scored every 20 min for a maximum of 3 h after KA administration. The maximum rating scale values reached by each animal over each 20 min interval were used to calculate the rating scale value (±SE) for each treatment group. Statistical analysis was performed by two-way repeated measures ANOVA followed by post hoc Holm-Sidak test. At the end of behavioral observation (3 h after KA), a subset of mice (WT, n=3; $En2^{-/-}$, n=5) was killed for *in situ* hybridization. Another subset of mice (WT, n=5; $En2^{-/-}$, n=7) was killed at 7 days after KA for histopathological analyses. An additional group of WT and $En2^{-/-}$ mice received saline injection and was used as controls for behavioral observations, *in situ* hybridization and histopathology.

In situ hybridization

Mice were killed at 3 h after KA injection, and brains were rapidly removed and frozen on dry ice. Coronal cryostat sections (20 μm thick) were fixed in 4% paraformaldehyde. Non-radioactive *in situ* hybridization was performed as previously described (Antonucci et al., 2008) using a digoxigenin-labeled c-*fos* and c-*jun* riboprobes (Bozzi et al., 2000). Signal was detected by alkaline phosphatase–conjugated anti-digoxigenin antibody followed by alkaline phosphatase staining. The specificity of the results was confirmed by the use of sense riboprobes which gave no detectable signal (not shown). Brain areas were identified according to Franklin and Paxinos (1997). To quantify the level of c-*fos* and c-*jun* mRNAs, digital images of three matching sections per animal,

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