Serotonin Hyperinnervation Abolishes Seizure Susceptibility in *Otx2* Conditional Mutant Mice

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The homeobox-containing transcription factor *Otx2* is crucially involved in fate determination of midbrain neurons. Mutant mice, in which *Otx2* was conditionally inactivated by a Cre recombinase expressed under the transcriptional control of the *Engrailed1* (*En1*) gene (*En1*^{cre/+}; *Otx2*^{flox/flox}), show a reduced number of dopaminergic neurons and an increased number of serotonergic neurons in the ventral midbrain. Despite these developmental anatomical alterations, *En1*^{cre/+}; *Otx2*^{flox/flox} adult mice display normal motor function. Here, we further investigated the neurological consequences of *Otx2* inactivation in adult *En1*^{cre/+}; *Otx2*^{flox/flox} mice. Adult *En1*^{cre/+}; *Otx2*^{flox/flox} mice showed increased serotonin (5-HT) levels in the pons, ventral midbrain, hippocampus (CA3 subfield), and cerebral cortex, as indicated by HPLC and immunohistochemistry. Conversely, SERT (5-HT transporter) levels were decreased in conditional mutant brains. As a consequence of this increased 5-HT hyperinnervation, *En1*^{cre/+}; *Otx2*^{flox/flox} mice were resistant to generalized seizures induced by the glutamate agonist kainic acid (KA). Indeed, prolonged pretreatment of *En1*^{cre/+}; *Otx2*^{flox/flox} mice with the 5-HT synthesis inhibitor *para*-chlorophenylalanine (pCPA) restored brain 5-HT content to control levels, fully reestablishing KA seizure susceptibility. Accordingly, *c-fos* mRNA induction after KA was restricted to the hippocampus in *En1*^{cre/+}; *Otx2*^{flox/flox} mice, whereas a widespread *c-fos* mRNA labeling was observed throughout the brain of *En1*^{cre/+}; *Otx2*^{flox/flox} mice pretreated with pCPA. These results clearly show that increased brain 5-HT levels are responsible for seizure resistance in *En1*^{cre/+}; *Otx2*^{flox/flox} mice and confirm the important role of 5-HT in the control of seizure spread.

Key words: serotonin transporter; pCPA; kainic acid; hippocampus; seizures; epilepsy

Introduction

The homeobox-containing transcription factor *Otx2* is required for regionalization, patterning, and neuronal differentiation in the midbrain (Simeone et al., 2002; Simeone, 2005). At the midbrain/hindbrain boundary, *Otx2* specifies identity and number of dopaminergic versus serotonergic progenitors by antagonizing the *Fgf8* (fibroblast growth factor 8) and *Shh* (sonic hedgehog) pathways and preventing ventral de-repression of the *Nkx2.2* transcription factor (Puelles et al., 2003, 2004; Prakash et al., 2006). In dopaminergic progenitors, *Otx2* is coexpressed with

Engrailed 1 (*En1*) (Puelles et al., 2004; our unpublished observations). In conditional mutant mice, in which *Otx2* was inactivated by a Cre recombinase expressed under the control of the *En1* promoter (*En1*^{cre/+}; *Otx2*^{flox/flox}), midbrain dopaminergic neurons were greatly reduced in number and most of their precursors underwent neurotransmitter fate switch, generating serotonin (5-HT)-positive neurons (Puelles et al., 2004). This alteration is maintained throughout life, because *En1*^{cre/+}; *Otx2*^{flox/flox} adult mice still display reduced dopamine (DA) and increased 5-HT levels in the striatum and cerebral cortex (Borgkvist et al., 2006).

Psychiatric and neurological conditions, such as affective disorders and epilepsy, depend on dysfunctions of the serotonergic system. As regards the role of 5-HT in epilepsy, it is known that agents that elevate extracellular 5-HT levels (such as 5-HT reuptake blockers) inhibit seizures, whereas 5-HT depletion results in the lowering of seizure threshold (Mazarati et al., 2005; Bagdy et al., 2007). The ventral midbrain and limbic system are crucially involved in 5-HT-mediated control of seizures (Bagdy et al., 2007).

In this study, we characterized the effects of serotonergic hyperinnervation in *En1*^{cre/+}; *Otx2*^{flox/flox} mice, investigating whether elevated 5-HT might contribute to decrease seizure sus-

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ceptibility in these animals. Seizures were evoked by systemic administration of the glutamate receptor agonist kainic acid (KA), a widely used model to study behavioral, anatomical, and cellular consequences of seizures in rodents (Lothman and Collins, 1981; Ben-Ari, 1985; Schauwecker and Steward, 1997). Here, we show that 5-HT hyperinnervation protects *En1*^{Cre/+}; *Otx2*^{flox/flox} mice against KA-induced seizures.

Materials and Methods

Animals. The generation and genotyping of *En1*^{Cre/+}; *Otx2*^{flox/flox} mutant mice have been reported previously (Puelles et al., 2003, 2004). In particular, the *En1*^{Cre/+} and the *Otx2*^{flox/+} mouse strains were maintained through continued breeding of at least seven generations with C57BL/6 × DBA2 F₁ mice. Then the two strains were mated to generate parental mice (*En1*^{Cre/+}; *Otx2*^{flox/+} and *Otx2*^{flox/flox}). *Otx2*^{flox/flox} mice were chosen as controls, because they do not show any anatomical or behavioral abnormality with respect to wild-type animals (Puelles et al., 2003; Borgkvist et al., 2006). Adult (6 months of age; weight, 20–35 g) mice of both sexes were used. 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).

Drug treatments. Drug-free *Otx2*^{flox/flox} and *En1*^{Cre/+}; *Otx2*^{flox/flox} mice (*n* = 3 per genotype) were used for initial 5-HT and 5-HT transporter (SERT) immunohistochemistry. To deplete 5-HT, mice (*n* = 5 per genotype) received *para*-chlorophenylalanine (pCPA) (4-chloro-L-phenylalanine hydrochloride; Sigma-Aldrich; 10 mg/ml stock in saline) twice a day (at ≈10:00 A.M. and 6:00 P.M.) at a dose of 100 mg/kg intraperitoneally for 3 consecutive days (Rantamäki et al., 2007). Five *Otx2*^{flox/flox} and five *En1*^{Cre/+}; *Otx2*^{flox/flox} mice received saline with the same schedule and served as controls. Sixteen hours after the last pCPA/saline injection, brains were dissected. One hemisphere was used for immunohistochemistry, and the other for HPLC. For seizure studies, 10 *Otx2*^{flox/flox} and 10 *En1*^{Cre/+}; *Otx2*^{flox/flox} mice received a single intraperitoneal injection of KA (Ocean Produce International; dissolved in saline) at 20 mg/kg. For pCPA plus KA treatments, mice (*n* = 10 per genotype), received the same dose of KA 16 h after the last pCPA injection. All experiments were performed blind to genotype and treatment.

Immunohistochemistry. Brains were fixed by immersion in 4% paraformaldehyde. Coronal or sagittal sections (50 μm; cut on a freezing microtome) were incubated overnight with anti-5-HT (1:5000; Sigma-Aldrich; or 1:200; Millipore Bioscience Research Reagents) or anti-SERT (1:5000; Calbiochem) polyclonal antibodies, diluted in a PBS solution containing 1% serum and 0.1% Triton X-100. Sections were then reacted with a biotinylated secondary antibody (Vector Laboratories) followed by avidin–biotin–peroxidase complex (ABC kit; Vector Laboratories) and diaminobenzidine reaction. Quantitative analyses of immunohistochemistry experiments was performed as reported in the supplemental material (available at www.jneurosci.org).

HPLC. 5-HT was measured according to Atkinson et al. (2006). Brain areas (pons/ventral midbrain, hippocampus, and cerebral cortex) were dissected on ice, weighed to the milligram sensitivity, and extracted with a buffer containing 8.2% ascorbic acid, 1.64% Na₂S₂O₅, and 0.83 M HClO₄. Extraction buffer volume (in microliters) corresponded to three times the weight in milligrams of the specimen. Homogenates were centrifuged (30 min; 18,000 rpm; 4°C), and supernatants were used as samples for HPLC. Standard solutions were prepared dissolving 5-HT and tryptophan (Sigma-Aldrich) in extraction buffer. Twenty microliters of samples or standards were injected into a Synergy Hydro-RP separation column, fitted with a C18 cartridge column (Phenomenex). The column was eluted isocratically (0.8 ml/min; 29°C) with mobile phase (100 mM ammonium acetate, pH 4.5; methanol; 12.5:1 v/v) in a Waters Alliance HPLC apparatus. Detection was performed with a Waters 474 scanning fluorescence detector (excitation and emission wavelengths, 290 and 337 nm, respectively), and data analysis was performed with Waters Millennium software. Values (±SE) were reported as picomoles of 5-HT per milligram of wet tissue. Statistical analysis was performed by one-way ANOVA followed by *post hoc* Tukey's test.

KA-induced seizures. Seizures were scored according to 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, seizure severity was scored every 20 min for 2 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.

In situ hybridization. Mice (*n* = 3 per genotype and treatment group, chosen among those used for behavioral analysis) were killed at 2 h after KA injection. Nonradioactive *in situ* hybridization was performed on brain coronal sections using a digoxigenin-labeled *c-fos* riboprobe, as described previously (Costantin et al., 2005).

Results

We first analyzed the distribution of 5-HT and SERT in the ventral midbrain and hippocampus of drug-free *En1*^{Cre/+}; *Otx2*^{flox/flox} mutant and *Otx2*^{flox/flox} control adult mice. Immunohistochemistry experiments confirmed the presence of 5-HT-positive neurons in the ventral midbrain of *En1*^{Cre/+}; *Otx2*^{flox/flox} but not *Otx2*^{flox/flox} mice (Fig. 1A) (Puelles et al., 2004). In the hippocampus, 5-HT staining was increased in the CA3 area of *Otx2* conditional mutant mice, compared with control mice (Fig. 1A). These findings were confirmed by quantitative analysis (see supplemental material, available at www.jneurosci.org). Conversely, SERT levels in serotonergic fibers were markedly reduced in these areas in *En1*^{Cre/+}; *Otx2*^{flox/flox} mice, when compared with control animals (Fig. 1B).

We next determined the 5-HT levels in different brain areas of *En1*^{Cre/+}; *Otx2*^{flox/flox} and *Otx2*^{flox/flox} mice (*n* = 5 per genotype). HPLC analysis in *Otx2* conditional mutant mice revealed a significant increase of 5-HT content in the pons/ventral midbrain, compared with control mice (Fig. 2A, saline-treated groups) (one-way ANOVA, *p* < 0.05; *post hoc* Tukey's test, control vs mutant, *p* < 0.05). A slight but not significant increase of 5-HT content was detected in the whole hippocampus of *En1*^{Cre/+}; *Otx2*^{flox/flox} compared with *Otx2*^{flox/flox} mice (one-way ANOVA, *p* > 0.05). According to our previous study (Borgkvist et al., 2006), 5-HT levels were also increased in the cerebral cortex of mutant mice (picomoles of 5-HT/milligram of tissue: saline-treated *Otx2*^{flox/flox}, 1.71 ± 0.19; saline-treated *En1*^{Cre/+}; *Otx2*^{flox/flox}, 4.8 ± 0.77; one-way ANOVA, *p* < 0.05; *post hoc* Tukey's test, *p* < 0.05). In both control and *Otx2* conditional mutant mice (*n* = 5 per genotype), prolonged treatment with the 5-HT synthesis inhibitor pCPA significantly reduced 5-HT levels in the pons, ventral midbrain, and hippocampus (Fig. 2A). pCPA also decreased 5-HT content in the cerebral cortex in both genotypes (picomoles of 5-HT/milligram of tissue: pCPA-treated *Otx2*^{flox/flox}, 0.98 ± 0.16; pCPA-treated *En1*^{Cre/+}; *Otx2*^{flox/flox}, 1.7 ± 0.5; one-way ANOVA, *p* < 0.05; *post hoc* Tukey's test, pCPA vs saline of same genotype, *p* < 0.05). 5-HT levels in pCPA-treated *En1*^{Cre/+}; *Otx2*^{flox/flox} mice did not significantly differ from those detected in saline-treated *Otx2*^{flox/flox} animals (Fig. 2A). 5-HT immunohistochemistry performed on brain sagittal sections from saline- and pCPA-treated *Otx2*^{flox/flox} and *En1*^{Cre/+}; *Otx2*^{flox/flox} mice confirmed these findings. According to our previous results (Fig. 1A,B) (Borgkvist et al., 2006), increased 5-HT staining was detected in several areas including ventral midbrain, basal forebrain, cerebral cortex (Fig. 2B), and pons (raphe nuclei) (Fig. 2B,C) of saline-treated *En1*^{Cre/+}; *Otx2*^{flox/flox} mutants, compared with saline-treated *Otx2*^{flox/flox} controls. Treatment with

Figure 1. 5-HT is increased and SERT is decreased in the ventral midbrain and hippocampus of *Otx2* conditional mutant mice. **A, B**, Images show coronal sections through the ventral tegmental area of the midbrain (top) and CA3 region of the hippocampus (bottom) from *Otx2*^{flox/flox} and *En1*^{Cre/+}; *Otx2*^{flox/flox} mice, stained with 5-HT (**A**) and SERT (**B**) antibodies. Scale bar, 150 μm.

Figure 2. 5-HT depletion in control and *Otx2* conditional mutant mice. **A**, HPLC determination of 5-HT content in the pons/ventral midbrain (top) and hippocampus (bottom) from *Otx2*^{flox/flox} and *En1*^{Cre/+}; *Otx2*^{flox/flox} mice, after a 3 d treatment with saline or pCPA. Data are reported as mean $\pm$ SE ($n = 5$ animals per group). * $p < 0.05$, *post hoc* Tukey's test. **B**, Representative low-magnification images showing 5-HT immunostaining on sagittal brain sections from control and *Otx2* conditional mutant mice, treated with saline or pCPA. Genotypes and treatments are as indicated. Abbreviations: bf, Basal forebrain; ctz, cerebral cortex; dr, dorsal raphe nucleus; p, pons; vmb, ventral midbrain. Scale bar, 3.4 mm. **C**, Representative high-magnification images showing 5-HT immunostaining in the dorsal raphe nucleus from control and *Otx2* conditional mutant mice, treated with saline or pCPA. Genotypes and treatments are as indicated. Scale bar, 150 μm.

pCPA markedly reduced 5-HT staining in all these areas in both genotypes (Fig. 2*B,C*). Quantitative analysis confirmed these findings (see supplemental material, available at www.jneurosci.org).

We next investigated whether increased 5-HT levels might alter seizure susceptibility in *Otx2* conditional mutant mice. Adult *En1*^{Cre/+}; *Otx2*^{flox/flox} and *Otx2*^{flox/flox} mice ($n = 10$ per genotype) received a single systemic injection of KA (20 mg/kg) and were observed for 2 h. KA treatment had a strong convulsant

effect in *Otx2*^{flox/flox} mice. All mice showed initial immobility, rapidly followed by repeated generalized (stage 4–6) seizures (Fig. 3). The mean latency to the first generalized seizure in KA-treated *Otx2*^{flox/flox} mice was 18.9 ± 8.7 min (Table 1). Progression of clinical signs was dramatically different in *En1*^{Cre/+}; *Otx2*^{flox/flox} animals (Fig. 3). Indeed, the trajectory in behavior score of *En1*^{Cre/+}; *Otx2*^{flox/flox} mice differed from that of control mice starting from 20 min after KA administration (two-way repeated-measures ANOVA, $p < 0.001$; *post hoc* Holm–Sidak

Figure 3. Resistance to KA-induced seizures in *Otx2* conditional mutant mice is abolished by 5-HT depletion. Graph shows the progression of behavioral changes over a 2 h observation period after KA in control and *Otx2* conditional mutant mice, with or without pCPA pretreatment. Genotypes and treatments are as indicated. Data are reported as mean seizure scores $\pm$ SE ($n = 10$ animals per group). ** $p < 0.001$, post hoc Holm–Sidak test, *En1*^{Cre/+}; *Otx2*^{flox/flox} versus the other three treatment groups.

test, *En1*^{Cre/+}; *Otx2*^{flox/flox} vs *Otx2*^{flox/flox} mice, $p < 0.001$). The majority of *Otx2* conditional mutant mice displayed only preconvulsive behaviors, never showing any sign of generalized seizure activity (Fig. 3). Only 1 of 10 *En1*^{Cre/+}; *Otx2*^{flox/flox} mice showed forelimb clonus with rearing and falling, followed by a single, brief tonic–clonic seizure. In this animal, latency to the first generalized seizure was 52 min (Table 1). *En1*^{Cre/+}; *Otx2*^{flox/flox} never showed any sign of generalized seizure activity also at later times (>2 h) after KA administration (data not shown). Depletion of endogenous 5-HT by pretreatment with pCPA in *Otx2* conditional mutant mice ($n = 10$) resulted in the occurrence of strong KA-induced generalized seizures, as observed in *Otx2*^{flox/flox} mice (Fig. 3). Indeed, seizure severity in *En1*^{Cre/+}; *Otx2*^{flox/flox} pretreated with pCPA was significantly different from that observed in the same mice without pCPA (two-way repeated-measures ANOVA, $p < 0.001$; post hoc Holm–Sidak test, *En1*^{Cre/+}; *Otx2*^{flox/flox} plus pCPA vs *En1*^{Cre/+}; *Otx2*^{flox/flox}, $p < 0.001$; *En1*^{Cre/+}; *Otx2*^{flox/flox} plus pCPA vs *Otx2*^{flox/flox}, $p > 0.05$). Latency to first generalized seizure in *Otx2* conditional mutant mice pretreated with pCPA was comparable with that observed in *Otx2*^{flox/flox} mice (Table 1). Pretreatment with pCPA in *Otx2*^{flox/flox} mice ($n = 10$) resulted in the same severity of KA-induced behavioral seizures as observed in *Otx2*^{flox/flox} mice without pCPA and *En1*^{Cre/+}; *Otx2*^{flox/flox} mice pretreated with pCPA (two-way repeated-measures ANOVA, $p > 0.05$; post hoc Holm–Sidak test, *Otx2*^{flox/flox} plus pCPA vs *Otx2*^{flox/flox} or *En1*^{Cre/+}; *Otx2*^{flox/flox} plus pCPA, $p > 0.05$). Saline-treated animals of all genotypes never showed any sign of seizure activity (data not shown).

We next used *c-fos* mRNA *in situ* hybridization to study the pattern of brain activation at 2 h after KA. A strong *c-fos* mRNA labeling was observed in the septum, caudate–putamen, cerebral cortex, amygdala, hypothalamus, and hippocampus of *Otx2*^{flox/flox} mice, whereas *c-fos* mRNA induction was restricted to the hippocampus in *En1*^{Cre/+}; *Otx2*^{flox/flox} mice (Fig. 4). Conversely, *En1*^{Cre/+}; *Otx2*^{flox/flox} mice pretreated with pCPA showed the same widespread *c-fos* mRNA labeling as observed in *Otx2*^{flox/flox} mice. Pretreatment with pCPA did not alter the pattern of KA-induced *c-fos* mRNA expression in *Otx2*^{flox/flox} mice

(Fig. 4). Saline-treated animals of both genotypes did not show any *c-fos* mRNA labeling throughout the brain (data not shown).

Discussion

In this study, we show that conditional inactivation of the *Otx2* gene in ventral midbrain results in increased 5-HT levels in several brain areas of *En1*^{Cre/+}; *Otx2*^{flox/flox} adult mice. Behavioral and gene expression analyses showed that mutant mice were markedly resistant to KA-induced seizures. Seizure resistance was attributable to increased 5-HT levels, because 5-HT depletion by pCPA fully reestablished KA seizure susceptibility in *En1*^{Cre/+}; *Otx2*^{flox/flox} mice.

A link between 5-HT and seizure inhibition was originally suggested by the observation that anticonvulsant drugs elevated brain 5-HT levels (Bonnycastle et al., 1957). Several studies then confirmed a crucial role of 5-HT in the control of seizures. It is now generally accepted that drugs elevating extracellular 5-HT levels [such as selective serotonin reuptake inhibitors (SSRIs)] exert a powerful antiepileptic action. For example, the anticonvulsant effect of the SSRI fluoxetine has been clearly demonstrated in a wide variety of experimentally induced seizure models, as well as in genetically epileptic animals. Conversely, depletion of brain 5-HT content is known to decrease the threshold to evoked seizures in several experimental paradigms (Bagdy et al., 2007). Our study confirms and expands this notion. Indeed, we showed that genetically induced increase of 5-HT levels results in marked protection against KA-induced seizures.

Different 5-HT receptors have been shown to modulate seizure susceptibility and development of epilepsy. More specifically, the 5-HT_{1A}, 5-HT_{2C}, 5-HT₃, and 5-HT₇ subtypes, which are all expressed in epileptogenic brain areas, are the most relevant in epilepsy (Bagdy et al., 2007). With respect to KA-induced seizures, the role of 5-HT_{1A} receptors has been clearly elucidated. Administration of 8-OH-DPAT [8-hydroxy-2-(di-*n*-propylamino)tetralin] (a specific 5-HT_{1A} agonist) reduces KA-evoked seizures in rats (Gariboldi et al., 1996), whereas increased lethality after KA seizures is observed in mice with targeted inactivation of the 5-HT_{1A} gene (Sarnyai et al., 2000). Mice lacking 5-HT_{2C} receptors also develop epilepsy (Tecott et al., 1995; Brennan et al., 1997). In KA seizure-resistant *En1*^{Cre/+}; *Otx2*^{flox/flox} mice, the role of 5-HT receptor signaling pathways remains to be elucidated.

Among the seizure models in which 5-HT plays a prominent role, DBA/2 mice have been well characterized. These mice are extremely prone to audiogenic or KA-induced seizures (Collins, 1972; Ferraro et al., 1995). Serotonin has been proposed to control seizure outcome in DBA/2 mice, because fluoxetine reduces respiratory arrest after audiogenic seizures (Tupal and Faingold, 2006). We used control and *Otx2* conditional mutant mice of a mixed DBA/2 $\times$ C57BL/6 background. *Otx2*^{flox/flox} control mice never showed spontaneous seizures, but displayed high susceptibility to seizures induced by 20 mg/kg KA, similarly to what observed in DBA/2 mice (Ferraro et al., 1995; McLin and Steward, 2006) and mice with a mixed DBA/2 $\times$ C57BL/6 background (Dell'agnello et al., 2007). Conversely, *En1*^{Cre/+}; *Otx2*^{flox/flox} mice showed a marked resistance to KA seizures, indicating a protective effect of 5-HT in the mixed DBA/2 $\times$ C57BL/6 background.

Seizure resistance in *En1*^{Cre/+}; *Otx2*^{flox/flox} mice was also confirmed by gene expression studies. A precise correlation exists between the pattern of *c-fos* induction and the appearance of generalized seizures after KA. Preconvulsive behaviors (stages 1–3 of Racine's scale) induce *c-fos* mainly in the hippocampus, whereas generalized seizures (stages 4–6) result in a widespread expression in several brain areas (Willoughby et al., 1997; Bozzi et

Table 1. Effect of 5-HT depletion on KA seizures in control and *Otx2* conditional mutant mice

	<i>Otx2</i> ^{flox/flox}	<i>En1</i> ^{Cre/+} ; <i>Otx2</i> ^{flox/flox}	<i>En1</i> ^{Cre/+} ; <i>Otx2</i> ^{flox/flox} + pCPA	<i>Otx2</i> ^{flox/flox} + pCPA
No. of animals with generalized (stage 4–6) seizures	10 of 10	1 of 10 ^a	10 of 10	10 of 10
Latency to first generalized (stage 4–6) seizure (min)	18.9 ± 8.7 ^b	52 ^c	25 ± 7.9	32.3 ± 12.7

^aThe number of animals with generalized seizures significantly differed between *En1*^{Cre/+}; *Otx2*^{flox/flox} and the other groups (*z* test, *p* < 0.001).
^bLatency to the first generalized seizure is calculated from the time of KA administration. Values (in minutes) are reported as mean ± SD. Latency did not differ between *Otx2*^{flox/flox}, *Otx2*^{flox/flox} + pCPA, and *En1*^{Cre/+}; *Otx2*^{flox/flox} + pCPA mice (one-way ANOVA, *p* = 0.062).
^cThe value reported refers to the only animal that showed a generalized seizure.

Figure 4. Effect of 5-HT depletion on *c-fos* mRNA expression in the brain of KA-treated control and *Otx2* conditional mutant mice. The panels show *c-fos* mRNA *in situ* hybridizations on coronal sections at the level of the caudate–putamen (top) and dorsal hippocampus (bottom) from representative control and *Otx2* conditional mutant mice (with or without pCPA pretreatment), 2 h after KA. Genotypes and treatments are as indicated. Abbreviations: amy, Amygdala; CPu, caudate–putamen; ctx, cerebral cortex; hip, hippocampus; ht, hypothalamus; sept, septum. Scale bar, 2 mm.

al., 2000). Accordingly, *En1*^{Cre/+}; *Otx2*^{flox/flox} mice never experienced generalized seizures after KA, showing *c-fos* mRNA induction restricted to the hippocampus. Conversely, *En1*^{Cre/+}; *Otx2*^{flox/flox} mice treated with pCPA, as well as *Otx2*^{flox/flox} mice, showed KA-induced generalized seizures and widespread induction of *c-fos* mRNA.

The ventral midbrain and limbic system are crucially involved in the serotonergic control of seizures. For example, endogenous 5-HT transmission in the substantia nigra is able to inhibit the spread of seizure activity generated in the limbic system (Pasini et al., 1996). In *En1*^{Cre/+}; *Otx2*^{flox/flox} mice, we detected increased levels of 5-HT in several brain areas, including the ventral midbrain, basal forebrain, cerebral cortex, and hippocampal CA3 subfield. Prolonged pretreatment of *En1*^{Cre/+}; *Otx2*^{flox/flox} mice with the 5-HT synthesis inhibitor pCPA restored brain 5-HT content to control levels and abolished seizure resistance in mutant mice. This indicates that increased availability of synaptic 5-HT is indeed protective against KA seizures in *En1*^{Cre/+}; *Otx2*^{flox/flox} mice. Increased synaptic availability of 5-HT was also

indicated by decreased SERT levels in the hippocampus and ventral midbrain of *En1*^{Cre/+}; *Otx2*^{flox/flox} mice. SERT decrease has been demonstrated to occur after prolonged elevation of 5-HT levels in mice (Mirza et al., 2007). Thus, in *Otx2* conditional mutant mice, decreased SERT levels might be a consequence of increased 5-HT innervation.

En1^{Cre/+}; *Otx2*^{flox/flox} mice show a strong reduction of DA cell number in the ventral midbrain (Puelles et al., 2004; Borgkvist et al., 2006). Therefore, it might be questioned that reduction of DA cells could also contribute to modify seizure susceptibility in these animals. DA, as 5-HT, is clearly involved in the control of epileptic seizures (Starr, 1996). A large series of studies strongly support the idea of an antiepileptic action of DA. Indeed, DA agonists inhibit convulsive seizures, both in experimental animals and humans. For example, the prototypical mixed D₁/D₂ receptor stimulant apomorphine has long been known to exert an antiepileptic action in humans (Starr, 1996). Mesolimbic DAergic pathways have been proposed to exert this inhibitory control (La Grutta and Sabatino, 1990; Starr, 1996). According to

this view, DA reduction in the mesolimbic system of *En1^{Cre/+}; Otx2^{fllox/fllox}* mice would be expected to exacerbate seizure severity. In contrast, spontaneous or KA-induced seizures were never observed in *En1^{Cre/+}; Otx2^{fllox/fllox}* mice, which instead displayed a marked resistance to KA. This suggests that, in this animal model, the effect of 5-HT hyperinnervation onto seizure control is more prominent than that of DA reduction.

Several studies suggest that there may be a link between epilepsy and depression, in which 5-HT would exert a crucial role. Epilepsy is often associated with depression in humans, and increased seizure susceptibility is accompanied by behavioral symptoms of depression in animal models (Mazarati et al., 2007). Moreover, serotonergic antidepressant drugs (such as fluoxetine) have anticonvulsant properties, and some antiepileptic drugs are effective in treating bipolar affective disorders (Jobe et al. 1999). We propose that *En1^{Cre/+}; Otx2^{fllox/fllox}* mutant mice, in which 5-HT hyperinnervation results in marked resistance to experimentally induced seizures, might serve as a novel genetic model to investigate the mechanisms underlying mood disorders.

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