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Developmental basis of seizure susceptibility: a focus on dopaminergic and serotonergic systems

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Doctor of Philosophy in Neuroscience

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INTRODUCTION

The role of major naurotransmitter pathways is epilepsy

Epilepsy is one of the most common neurological disorders, affecting about 1% of human population worldwide. Epilepsy is characterized by the repeated occurrence of sudden, transitory and localized bursts of electrical activity, known as seizures. Seizures are temporary alterations in brain functions due to abnormal electrical activity of a group of brain cells that result in a wide variety of clinical and sub-clinical symptoms.

A large body of experimental evidence suggests that overactivity of the excitatory neurotransmitter glutamate, or reduced activity of the inhibitory neurotransmitter GABA, is central to the process of epileptogenesis. However, all major neurotransmitter pathways regulate overall brain excitability and are involved in epileptogenesis. Specifically, monoamines represent a group of neuroactive substances that are capable of regulating the initiation and spread of seizure activity. Dopamine (DA) may be crucially involved in propagation of seizure and control of seizure threshold (Starr 1996). Serotonin (5-hydroxytryptamine, 5-HT) is another monoaminergic transmitter crucially involed in seizure propoagation. Different types of 5-HT receptors are present on the cortical and/or hippocampal glutamatergic or GABAergic neurons or terminals, where they can cause a significant shift in excitability in most networks involved in epilepsy (Badgy *et al.* 2007). Classical pharmacological studies clearly showed that both DA and 5-HT may have potent anti-convulsant effects, acting through specific receptor pathways.

There is a substantial body of clinical data in support of an antiepileptic action of dopamine in man. It is widely accepted that agents which increase dopamine level (dopamine agonists) are considered as an anticonvulsants and reduce the seizure threshold, while agents which block dopamine action (dopamine antagonists) are considered as a proconvulsants. Consistent with that, clinical data not only indicate that high dopaminergic activity in the brain suppresses seizure activity, but also that a

reduction of dopaminergic tone is pro-epileptogenic (Starr, 1996). This led to hypothesize that dopamine is an important suppressor of the processes which govern the genesis and propagation of seizures.

Studies in animal models and analysis of dissected tissue from epileptic patients provide evidence that endogenous 5-HT, the activity of its receptors, and pharmaceuticals with serotonin agonist and/or antagonist properties play a significant role in the pathogenesis of epilepsies (Bagdy *et al.* 2007). The effects of genetic manipulation and pharmacological intervention (including the effects of subtype-selective receptor agonists and antagonists) on the development of seizure and epileptic activity have been clearly characterized (see below). Moreover, anti-epileptic drugs elevate and/or stimulate basal 5-HT levels and/or release, (Okada *et al.* 1992; Dailey *et al.* 1996; Ahmad *et al.* 2005).

Dopamine synthesis and Dopaminergic transmission

Dopaminergic pathways are neural pathways in the brain by which dopamine is spread to a range of different destinations in the brain. There are four major central dopamine-containing pathways, which are described in Figure 1.

1) The nigrostriatal pathway, in which substantia nigra (SN) neurons innervate the striatum. This pathway is involved in movement control.
2) The mesocortical pathway, which links the ventral tegmental area (VTA) to medial prefrontal, cingulate and entorhinal cortices. This pathway is involved in motivational and emotional responses.
3) The mesolimbic pathway, composed of VTA cells projecting to the nucleus accumbens and other limbic areas which includes the amygdala and the hippocampus. This pathway is significantly involved with reward and pleasure response.
4) The tuberoinfundibular system, which projects from arcuate and periventricular nuclei of the hypothalamus to the pituitary gland and is involved in the control of neuroendocrine function.

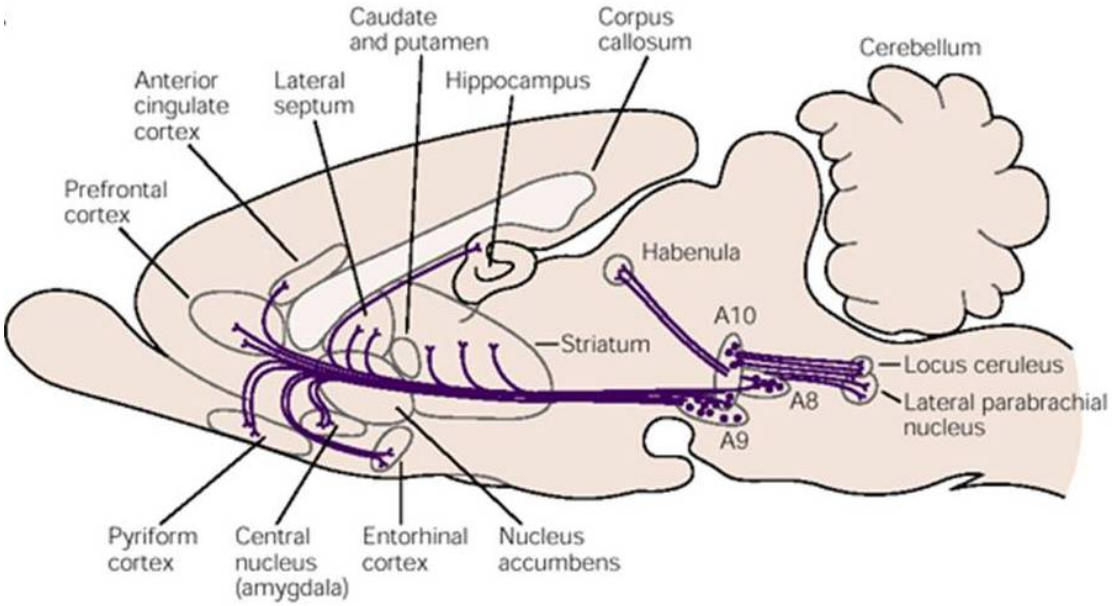

Figure 1. Schematic representation of major dopaminergic pathways in rodents**,** originate from groups of cells in the rostral areas of the brain (Kandel, Principles of Neural Science, 4th edition)

All these pathways are involved in several neurological, psychiatric and neuroendocrine diseases (Figure 2). Reduced dopamine levels in the nigrostriatal pathway, resulting from selective loss of SN cells, are the primary cause of Parkinson's disease. Reduced function of the nigrostriatal system (as a consequence of striatal degeneration) is also typical of Huntington's disease. Conversely, increased dopamine signalling in the striatum and frontal lobes has been implicated in attention-deficit–hyperactivity disorder (ADHD; Madras *et al*. 2005). Increased levels of dopamine in the mesolimbic pathway, namely in the nucleus accumbens, represent the neurobiological substrate of the rewarding properties of all drugs of abuse, and also contribute to appetite disorders (Volkow and Wise 2005).

Altered dopamine signalling in the limbic system has also been implicated in epilepsy (Starr, 1996; Bozzi *et al.* 2000) and, more recently, in depression (Park *et al.* 2005). Finally, reduced dopamine signalling to the hypophysis is clearly implicated in the aetiology of pituitary tumours (Iaccarino *et al.* 2002).

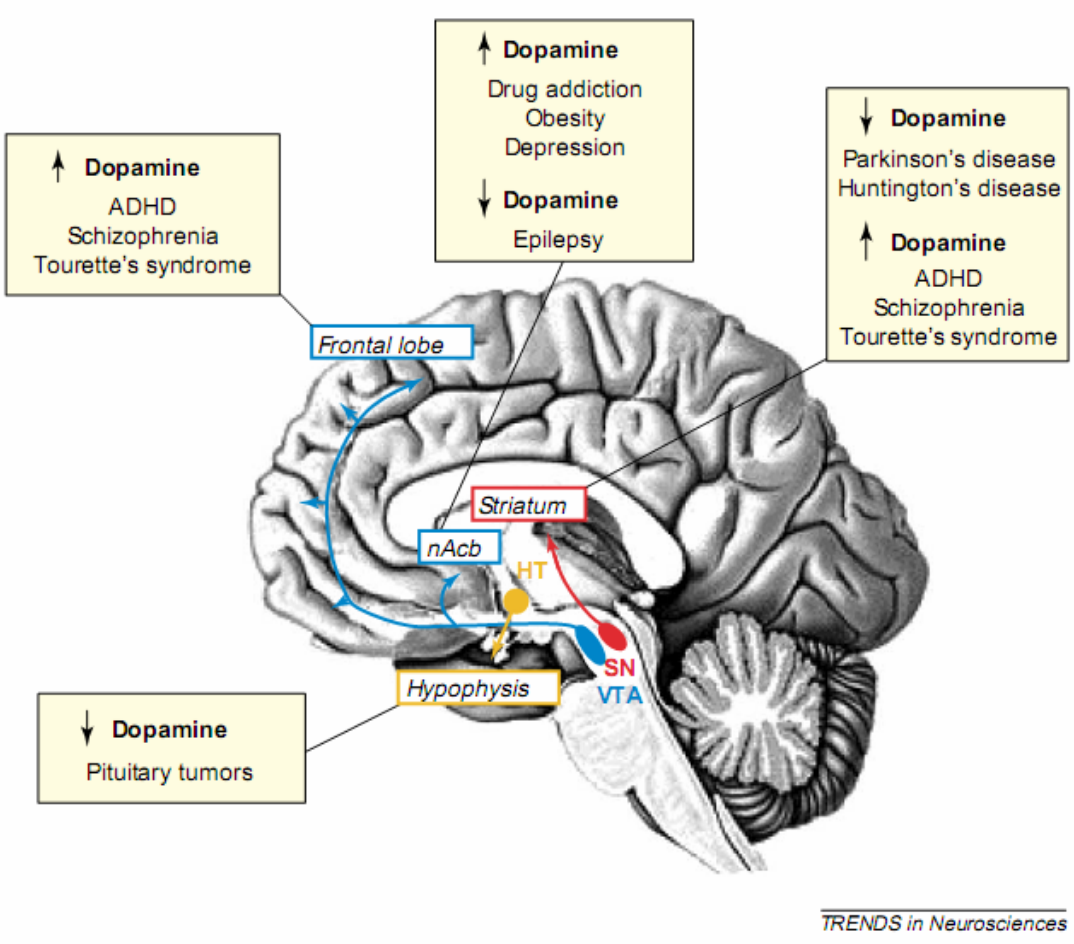

Figure 2. Alterations of dopamine systems in neurological, neuroendocrine and psychiatric diseases. Dopamine pathways are shown in different colours (red, nigrostriatal; blue, mesocortical and mesolimbic; orange, tuberoinfundibular). Altered levels of dopamine function in specific areas are indicated for the different pathologies. Abbreviations: ADHD, attention-deficit–hyperactivity disorder; HT, hypothalamus; nAcb, nucleus accumbens; SN, substantia nigra; VTA, ventral tegmental area (from Bozzi and Borrelli, 2006).

Dopamine synthesis, like that of all catecholamines, originates from the amino acid precursor tyrosine, which must be transported across the blood brain barrier into the dopamine neuron. Dopamine is synthesized in the body (mainly by nervous tissue and the medulla of the adrenal glands) first by the hydroxylation of the amino acid L-tyrosine to L-DOPA via the enzyme tyrosine 3-monooxygenase, also known as tyrosine hydroxylase, and then by the decarboxylation of L-DOPA by aromatic L-amino acid decarboxylase (which is often referred to as dopa decarboxylase). This latter

enzyme turns over so rapidly that DOPA levels in the brain are negligible under normal condition. Because of the high activity of this enzyme and the low endogenous levels of DOPA normally present in the brain, it is impossible to enhance dramatically the formation of dopamine by providing this enzyme with increased amount of substrate. Since tyrosine hydroxylase is the rate-limiting enzyme in the biosynthesis of dopamine, this enzyme sets the pace for the formation of dopamine synthesis and physiological regulation (Figure 3).

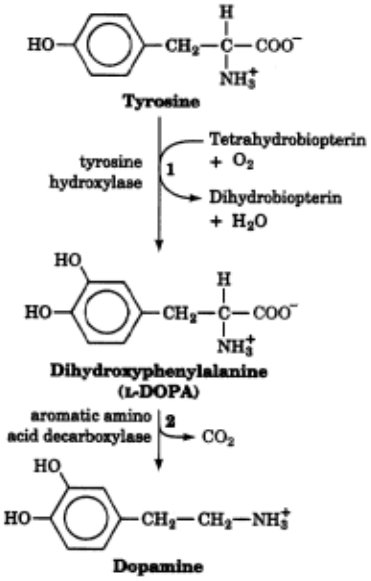

Figure 3. Biosynthesis of Dopamine- Tyrosine, an amino acid abundant in dietary proteins, is first hydroxylated into L-DOPA. The cytostolic enzyme, tyrosine hyroxylase, catalyses this conversion and is normally the rate-limiting step in dopamine biosynthesis. Subsequently, aromatic amino acid decarboxylase (dopa-carboxylase) catalyses the conversion of L-DOPA to dopamine (http://sprojects.mmi.mcgill.ca/gait/parkinson/biochemistry.asp)

In dopaminergic neurons, dopamine is transported from the cytoplasm to specialized storage vesicles. Upon the arrival of an action potential which triggers subsequent exocytosis, vesicles discharge the neurostransmitters into the synapse. Dopaminergic terminals possess transporters (dopamine transporter, DAT) that are critical in terminating transmitter action and in maintaining transmitter homeostatsis through DA reuptake (figure 4). Under normal conditions, potent, high-affinity membrane carriers recycle dopamine that has been released into synaptic cleft by actively pumping extracellular dopamine back into the nerve terminal. (Elsworth *et al.* 2002).

The dopamine receptor family contains five members that, according to structural and pharmacological similarities, are divided into two sub- families: the D1-like family, comprising D1 and D5 receptors; and the D2-like family, which includes D2, D3 and D4 receptors (Cooper 1996; Jackson *et al.* 1994).

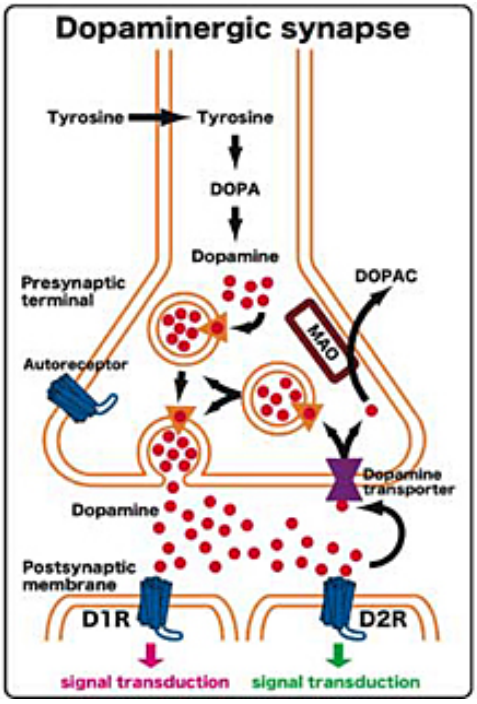

Figure 4. Schematic drawing of dopaminergic neurotransmission- The key steps in synthesis and degradation of dopamine (http://www.nibb.ac.jp/en/sections/sasaoka.html).

DA and epilepsy.

DA has long been postulated to have an anti-epileptic action. The anti-convulsant properties of apomorphine (a prototypic DA agonist) were first described more than one century ago. Seizure inhibition has been also observed in patients administered amphetamines or antiparkinsonian drugs such as pergolide and bromocriptine, which are potent D2 agonists, which all stimulate dopaminergic transmission. For example Gatterau and coworkers (1990) found that pergolide gave complete relief against temporal lobe epilepsy when administered to patients in a daily dose of 25-50 pg for 8 months. Moreover, the protection lasted for a further 27 months after discontinuing the treatment. Mauro *et al.* (1986) gave bromocriptine in conjunction with

the MAO-B (Monoamine Oxidase-B) inhibitor selegiline to patients suffering from Lafora's disease and showed it markedly reduced the frequency of generalised convulsive seizures and myoclonic jerks.

There are several reports dealing with the influence of 6-OHDA (6- hydroxydopamine) treatment on seizure sensitivity during early postnatal development. London and Buterbaugh (1978) showed that intracisternal 6-OHDA modified tonic pentylenetetrazol convulsions in young rats. Also, MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) selectively depleted brain dopamine (but not noradrenaline or 5-hydroxytryptamine) and abolished strychnine and electroshock-induced seizures in mice (Fariello *et al.* 1987).

The use of dopaminergic ligands specific for the different subclasses of DA receptors allowed to demonstrate that DA has an anti-epileptic action also in a wide variety of animal models (Starr 1993, 1996). In particular, these studies illustrated the opposite actions of D1-like and D2-like receptors in the regulation of seizure activity. The physiological balance of DA activity at these two different receptors would be crucial for determining the response to seizure-promoting stimuli: activation of D1-like receptors is generally pro-convulsant, whereas D2-like receptor stimulation can block seizures. More recently, studies performed on different dopamine receptor knockout mouse lines confirmed these findings (Bozzi *et al.* 2006; Bozzi *et al.* 2002; Bozzi *et al.* 2000; O'Sullivan *et al*. 2008). The limbic system is crucially involved in the dopaminergic control of epileptic seizures. Indeed, limbic areas of the brain receive dopaminergic innervation (Verney *et al.* 1985) and express different types of DA receptors (Jackson *et al.* 1994). Indeed, a high abundance of DA D1 and D2 receptors has also been detected in amygdala (Camps *et al*. 1990, Palacios and Pazos, 1987). DA D2 receptor levels are medium to high in laminae V and VI in both cingulate and temporal cortex and in the entorhinal cortex (Bouthenet *et al.* 1987), while mRNA for the DA D5 receptor has been detected mainly in hippocampal (Meador-Woodruff *et al.* 1992).

5-HT synthesis and 5-HT transmission

The principal centres for serotonergic neurons are the rostral and caudal raphe nuclei. From the rostral raphe nuclei axons ascend to the cerebral cortex, limbic regions and specifically to the basal ganglia. Serotonergic nuclei in the brain stem give rise to descending axons, some of which terminate in the medulla, while others descend the spinal cord (Figure 5). Serotonin plays a role in many brain processes, including regulation of body temperature, sleep, mood, appetite and pain. Problems with the serotonin pathway can cause obsessive-compulsive disorder, anxiety disorders, and depression.

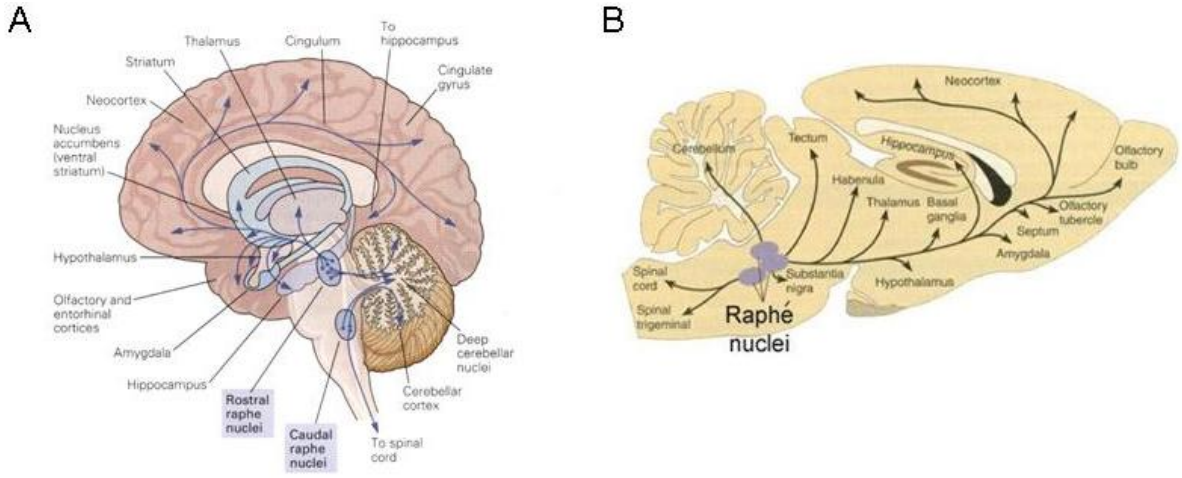

Figure 5. The major human (A) and rodents (B) serotonergic pathways arise in the raphe nuclei (Adapted from Heimer 1995 and Kandel, Principles of Neural Science, 4th edition).

Serotonin is synthesized from the amino acid L-tryptophan (Figure 6). Transformation of tryptophan into serotonin involves two steps:

1) Hydroxylation in 5-hydroxytryptophan catalyzed by tryptophan hydroxylase (TPH).
2) Decarboxylation of 5-hydroxytryptophan is catalyzed by L-aromatic amino acid decarboxylase (DDC).

TPH-mediated reaction is the rate-limiting step in the pathway. TPH has been shown to exist in two forms: TPH1, found in several tissues, and TPH2, which is a brain-specific isoform. In the brain, serotonin biosynthesis depends on the quantity of tryptophan

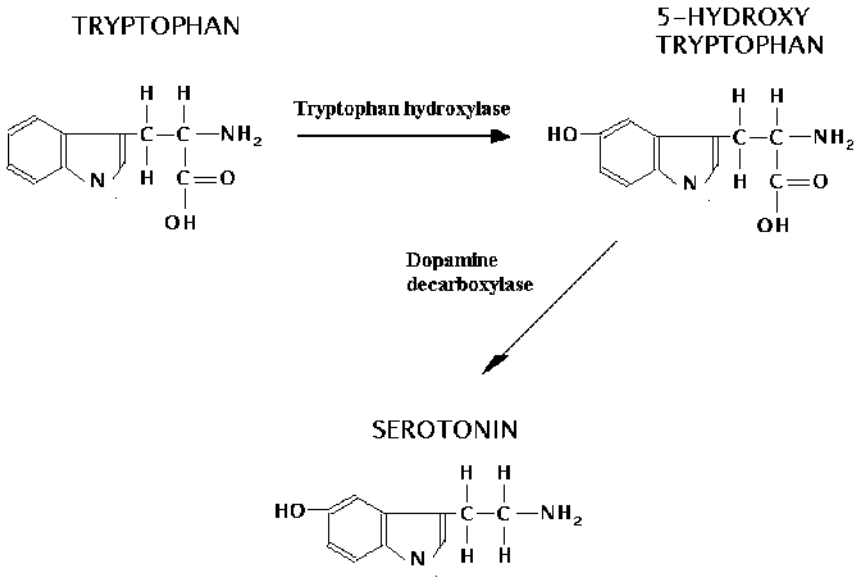

Figure 6. Serotonin is synthesized from the amino acid L-tryptophan by a short metabolic pathway consisting of two enzymes: tryptophan hydroxylase (TPH) and amino acid decarboxylase (DDC). (http://herkules.oulu.fi/isbn9514267672/html/i43346.html)

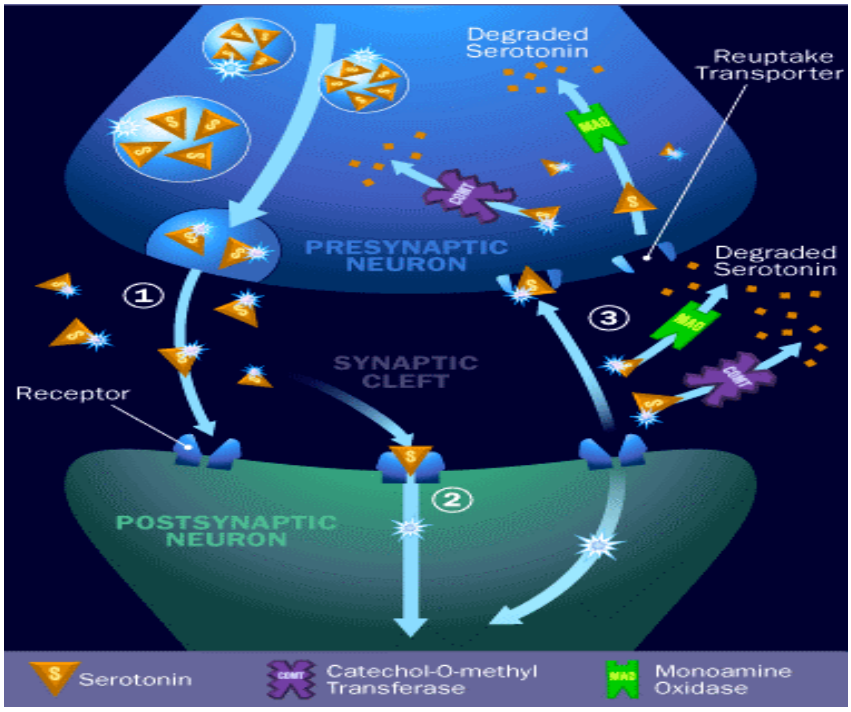

Figure 7. Serotonergic transmission (http://health.howstuffworks.com/nerve5.htm)

which crosses the blood-brain barrier. Only free plasma tryptophan penetrates into the brain.

5-HT receptors are a group of G protein-coupled receptors (GPCRs) and ligand-gated ion channels (LGICs) found in the central and peripheral nervous system.There are 7 different types of 5-HT receptors (5-HT1 to 5-HT7) and each of 7 classes contains many receptor subtypes.

Serotonergic action is terminated primarily via uptake of 5-HT from the synapse. This is through the specific monoamine serotonin transporter (SERT), on the presynaptic neuron. Various agents can inhibit 5-HT reuptake including tricyclic antidepressants (TCAs) and selective serotonin reuptake inhibitors (SSRIs) (Figure 7).

5-HT and epilepsy

The idea that there may be a link between 5-HT and seizure inhibition was first suggested as early as 1957 (Bonnycastle *et al*.). In this study, a series of anticonvulsants, including phenytoin, were shown to elevate brain 5-HT levels. In recent years, there has been increasing evidence that serotonergic neurotransmission can modulate seizures in a wide variety of experimental models. It is now generally accepted that drugs elevating extracellular 5-HT levels (such as 5-hydroxytryptophan or selective serotonin reuptake inhibitors, SSRI) exert a powerful antiepileptic action against both focal (limbic) and generalized seizures (Löscher 1984; Prendiville *et al.* 1993; Yan *et al.* 1994). 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 by para-chloroamphetamine (PCA, a selective neurotoxin for 5-HT neurons) or para-chlorophenilalanine (pCPA, an inhibitor of 5-HT synthesis) can lower seizure threshold, increasing the severity of limbic status epilepticus (Bagdy *et al.* 2007; Mazarati *et al.* 2005).

5-HT receptors are expressed in almost all networks involved in epilepsies. Audiogenic seizures are the best known defect caused by genetic manipulation of a 5-HT receptor subtype which provides a robust model for examination of the serotonergic mechanism in epilepsy. Mutant mice lacking the 5-HT2C receptor subtype are extremely susceptible to audiogenic seizures and are prone to spontaneous death after seizures, suggesting that serotonergic neurotransmission mediated by 5-HT2C receptors suppresses neuronal network hyperexcitability and in turn seizure activity (Tecott *et al.* 1995; Brennan *et al.* 1997; Applegate and Tecott 1998). 5-HT1A receptor knockout mice display lower seizure threshold and higher lethality in response to glutamate agonist kainic acid (KA) administration. Furthermore, 5-HT1A knockout mice demonstrate impaired hippocampal-dependent learning and enhanced anxiety related behaviours (Sarnyai *et al.* 2000; Parsons *et al.* 2001). The areas which are crucially involved in the serotonergic control of seizures are the ventral midbrain and limbic system such as amygdala and hippocampus.

Fourteen mammalian 5-HT receptor subtypes are currently recognized, and these have been classified into seven receptor families on the basis of their structural, functional and, to some extent, pharmacological characteristics (Bradley *et al.* 1986; Hoyer *et al.* 1994). Among these receptors, the 5-HT1A, 5-HT2C, 5-HT3 and 5-HT7 subtypes, which are all expressed in epileptogenic brain areas (mainly, cerebral cortex and/or hippocampus), are the most relevant in epilepsy (Bagdy *et al.* 2007). 5-HT1A receptors are located both postsynaptically to 5-HT neurons (in the forebrain regions) at the level of the soma and dentrites in the mesencephalic and medullary raphe nuclei, cortical pyramidal neurons as well as pyramidal and granular neurons of the hippocampus (Francis *et al.* 1992). 5-HT2C binding sites are widely distributed and present in choroid plexus, areas of the cortex (olfactory nucleus, pyriform, cingulated and retrospenial), limbic system (nucleus accumbens, hippocampus and amygdala) and the basal ganglia (caudate nucleus and substantia nigra). 5-HT3 receptors are found in the nervous system both centrally and peripherally. The highest density of 5-HT3 receptors in the brain is found in the nuclei of the brainstem. Lower densities of 5-HT3-binding sites are found in the cortex and areas of limbic region such as hippocampus, amygdala,

and in medial nucleus of the habenula. 5-HT7 receptor expression is relatively high within the thalamus, hypothalamus and hippocampus with generally lower levels in areas such as the cerebral cortex and amygdala (To *et al.* 1995; Gustafson *et al.* 1996; Stowe and Barnes 1998).

Indeed, the role of at least 5-HT1A, 5-HT2C, 5-HT3 and 5-HT7 receptor subtypes in epileptogenesis and/or seizure propagation has been described. These receptors are present on cortical and/or hippocampal glutamatergic or GABAergic neurons or terminals (Bagdy *et al.* 2007). For example, 5-HT1A receptor knockout mice display lower seizure thresholds and higher lethality in response to kainic acid administration (Sarnyai *et al.* 2000). Growing body of evidence suggest that 5-HT1A receptors may have an inhibitory role in the generation of hippocampal seizures and it depends on 5-HT1A postsynaptic receptors. Neuroanatomical evidence shows a dense innervation of 5-HT fibres to the hippocampus mainly originating from the median raphe forebrain nucleus (Azmitia & Segal, 1978).

Inhibition of epileptiform bursts was also achieved with the selective 5-HT1A agonist, 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT, a specific 5-HT1A agonist). They found that these effects were completely antagonized by WAY-100135, a 5-HT1A receptor antagonist (Salgado and Alkadhi, 1995). Administration of 8-OH-DPAT is able to reduce experimentally induced seizures in rats (Gariboldi *et al.* 1996). In general, hyperpolarization of glutamatergic neurons by 5-HT1A receptors and depolarization of GABAergic neurons by 5-HT2C receptors as well as antagonists of 5-HT3 and 5-HT7 receptors decrease the excitability in most networks involved in epilepsies (Bagdy *et al.* 2007).

Embryonic development of Dopaminergic and Serotonergic neurons:

The embryonic development of the Central Nervous System (CNS) requires an orchestrated series of events tightly regulating the patterning and regionalization of the neural tube, as well as the proliferation, survival and differentiation of distinct neuronal populations. All these events are controlled by cascades of activation of transcription

factors that regulate the expression of specific subsets of genes in restricted regions and neuronal populations of the developing CNS. Among these transcription factors, homeobox-containing proteins play a crucial role, and altered expression of these factors can impact embryonic as well as adult CNS functions. In particular, homeobox-containing genes have been described to crucially regulate differentiation of dopaminergic and serotonergic neurons during brain development. In the adult brain, dopaminergic and serotonergic neurons, respectively located in midbrain and hindbrain regions, diffusely innervate several forebrain areas, contributing to regulate several physiological functions including brain excitability. DA neurons are divided into ten distinct groups. The most prominent ones reside in the ventral midbrain (called A8, A9 and A10), and in the diencephalon (groups A11-A15). The telencephalon contains two smaller groups of DA neurons, and these are restricted to the olfactory bulb (A16 group) and retina (A17 group). Groups A1-A7 are noradrenergic. Mammalian 5-HT neurons are classically divided from anterior to posterior into nine cell groups (B9–B1, respectively). The more rostral 5-HT groups (B9-B5) reside in the midbrain and rostral hindbrain, whereas groups B4-B1 are located more caudally. Rostral group accounts for 85% of all serotonergic neurons in the brain. The most rostral serotonergic neurons in the brainstem are located in the ventral tegmental area (Cordes 2005).

Understanding the embryonic development of these neuronal subtypes is crucial to elucidate their physiological function in the adult brain. In the mammalian nervous system, individual populations of neurons develop in a stereotypic position identified by their coordinates along the antero-posterior (A/P) and dorso-ventral (D/V) axes of neural tube (Hynes *et al.* 1999; Tanabe *et al.* 1996). Three organizing centers, the mid-hindbrain boundary (MHB or isthmus), the floorplate (FP), and the anterior neural ridge (ANR) control regionalization of the two main axes and specify the location and the cell fate of specific neuronal population within the brain fate map (Rubenstein *et al.* 1994). This is also true for dopaminergic (DA) and serotonergic (5-HT) neurons localized in caudal midbrain and rostral hindbrain, respectively (Hynes *et al.* 1999).

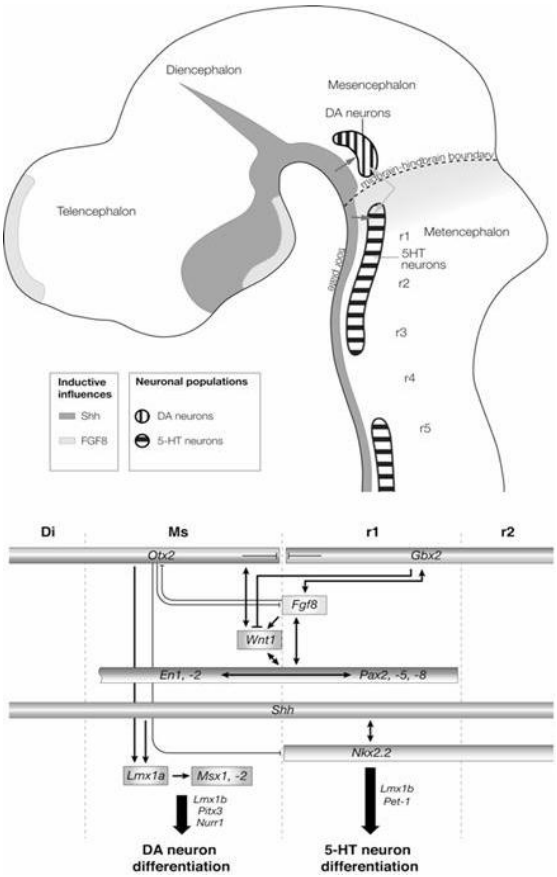

Figure 8. Gene regulatory networks involved in DA and 5-HT neuron differentiation.
(Top) Patterning signals in the developing brain. Sagittal view of an E11 mouse embryo neural tube; anterior is on the left. Expression of the secreted factors fibroblast growth factor 8 (Fgf8), Wnt1 and sonic hedgehog (Shh) is depicted at the MHB, in the anterior neural ridge and ventral diencephalon and within the floor/basal plate of the spinal cord, hindbrain, midbrain and caudal forebrain. Mesencephalic dopaminergic (DA) neurons are induced by a combination of Fgf8 and Shh (arrows). 5-HT neurons are specified by a combination of the same factors but they require an early inductive signal (Fgf4, not shown) derived from the anterior mesoderm during gastrulation. (Bottom) Gene expression patterns participating to DA and 5-HT neuron differentiation; anterior is on the left. Gbx2 expression maintains Fgf8 expression, whereas Otx2 and Gbx2/Fgf8 regulate each other negatively. Concomitantly, the expression territories of Fgf8, Wnt1, Engrailed and Pax genes become interdependent and establish a positive regulatory loop that is necessary to maintain MHB identity. The mid-diencephalic border is positioned by negative cross-regulations of Engrailed/Pax and Pax6 (not shown), whereas Fgf8 exerts a negative influence on the caudal expression of Hox genes (not shown). Later on, Shh induces the expression of Lmx1a and Msx1/2. While Lmx1a is sufficient to induce DA cell differentiation of ventral progenitors cells and induces the expression of Msx1/2, Msx1/2 is involved in the repression on the lateral progenitors cell fate (not shown). Midbrain DA neurons are specified dorso-ventrally by Shh signaling and antero-posteriorly by Otx2 signals, while 5-HT cells originate from precursors lacking the Otx2 signal. Shh signaling induces the expression of Nkx2.2, which is then essential for specification of 5-HT neurons and ventral progenitor identity, conferring competence to become 5-HT neurons. Once positioning and identity of the neuronal precursors are determined, specific differentiation programs are activated in DA (Lmx1b, Pitx3, Nurr1) and 5-HT neurons (Lmx1b and Pet-1). Adapted from Prakash & Wurst 2004, Wurst & Bally-Cuif 2001, Tripathi et al. 2010. See text for details. Abbreviations: Di, diencephalons; Ms, mesencephalon; r, rhombomeres.

Regionalization of midbrain/hindbrain territory.

The MHB is anatomically characterized as a neuroepithelial constriction between midbrain and hindbrain; however, as an organizing center, the MHB is the tissue that has the ability to recreate pattern when transplanted to a different region of the neural tube. Anatomical boundaries of this functional center have not yet been determined, so investigators have used genes that either are expressed specifically or have borders that terminate in the region at the early embryonic ages to define it. For this reason the borders of the MHB are only defined with the use of expression patterns of specific genes, which delineate its competence territory (Figure 8). These genes are expressed in the junction (e.g. En1, En2, Pax2, Pax5, Pax8, and Fgf8, Fgf17 and Fgf18), or cover a broad domain that terminate at the boundary between the mid- and hindbrain (e.g. Otx2 and Gbx2).

On the basis of expression of the multiple MHB-associated genes, it has been determined that the MHB initially covers a broad region within the neural plate, occupying territories in both the presumptive midbrain and presumptive hindbrain. Between the headfold stage (>1 somite) and mid-gestation, it appears that this region gradually diminishes in size, subsequently occupying an area between the midbrain and hindbrain. By embryonic stage 7.5 (E7.5) in mouse, the transcription factors Otx2 and Gbx2 are expressed in a complementary fashion in the embryo: the border along their expression territories delineates the future junction between mesencephalon and metencephalon, the MHB (Figure 9). Slightly later, at E8, the transcription factor Pax2 and the secreted molecule Wnt1 are expressed in broad, overlapping domains (Rowitch & McMahon, 1995). Wnt1 expression is largely restricted to the Otx2-positive territory, whereas Pax2 expression crosses the Otx2/Gbx2 border (Bally-Cuif, 1995). Shortly after, the transcription factors Engrailed 1 (En1) (at the 1-somite stage), Engrailed 2 (En2) (at the 3–5-somite stage) (Davis & Joyner 1988; Davis *et al.* 1988) and Pax5 (at the 3/5-somite stage) (Asano & Gruss, 1992) are expressed across the Otx2/Gbx2 border. The secreted factor fibroblast growth factor 8 (Fgf8) is similarly switched on at the 3–5 somite stage but is restricted to the caudal, Gbx2-positive side of the mes-metencephalic junction (Crossley & Martin, 1995).

Otx1 and Otx2 are expressed rostral to the MHB (Simeone 1992, 1993; Millet 1996). Some others, such as Gbx2, are caudal to this boundary (Bulfone *et al.* 1993; von Bubnoff *et al.* 1995; Wassarman *et al.* 1997; Hidalgo-Sánchez *et al.* 1999), whereas others, such as En1 and En2 (Davis et al. 1988; Gardner *et al*. 1988) and three members of the Pax family (Pax2, Pax5 and Pax8; Asano and Gruss, 1992) are expressed at both sides of the boundary, forming a decreasing gradient in rostral and caudal directions (Hidalgo-Sánchez *et al.* 1999).

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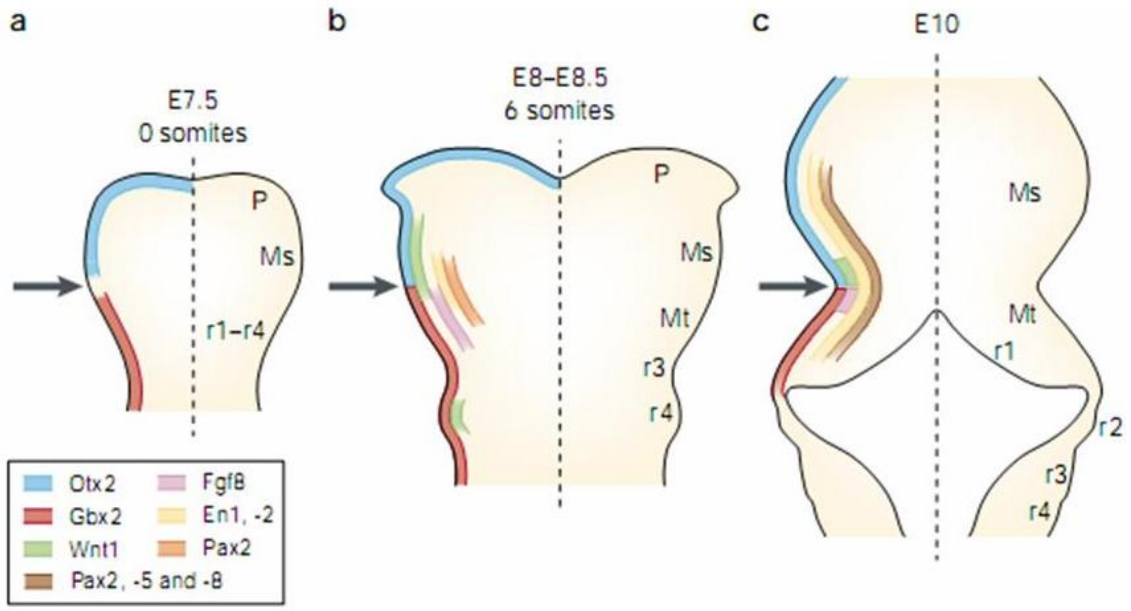


Figure 9. Dynamics of gene expression patterns at the mid-hindbrain border.
Dorsal views of the mouse embryonic neural plate at a) 0-somite stage, b) 6-somite stage and c)| E10 stage. Anterior is on the top. a) At the end of gastrulation (0 somites), the neural plate is broadly subdivided into an anterior domain that expresses Otx2 and a posterior domain that expresses Gbx2. The expression patterns of both genes meet at the mid-hindbrain border and form decreasing gradients in opposite directions. b) At 6 somites, the posterior border of Otx2 expression and the anterior border of Gbx2 expression have sharpened and abut each other. Wnt1 expression is initiated in the mesencephalon, and En1 (quickly followed by En2) and Pax2 are turned on across the Otx2–Gbx2 border. Slightly later, Fgf8 expression is recruited to the caudal side of the Otx2–Gbx2 border. c) At E10, the Otx2–Gbx2 border identifies the midbrain/hindbrain boundary. The expression of Wnt1 and Fgf8 has become restricted to narrow rings encircling the neural tube on either side of this boundary. The domains of En1 and Pax2 expression, which still overlap the boundary, have also become narrower, whereas En2, Pax5 and Pax8 are expressed across most of the mid-hindbrain domain. (En, engrailed; Fgf8, fibroblast growth factor 8; Gbx2, gastrulation brain homeobox 2; Ms, mesencephalon; Mt, metencephalon; Otx2, orthodenticle homologue 2; P, prosencephalon; Pax, paired box; r, rhombomeres; hatched line, axis of symmetry). An arrow to the left of each panel indicates the position of the midbrain/hindbrain boundary. (Wurst & Bally-Cuif, 2001)

At early stages Fgf8, Wnt1 and Otx2 are expressed in the caudal midbrain regions that give rise to midbrain DA neurons. In contrast, Fgf8 and Gbx2, but not Wnt1, are expressed in the region that gives rise to rostral 5-HT progenitors (Figure 8). The transcription factors Engrailed1 (En1) and Engrailed2 (En2) are instead expressed in both caudal midbrain and anterior hindbrain. The expression domain of each gene reflects the role the gene plays in the formation of this territory. Otx2 and Gbx2 expression domains are restricted to the anterior and posterior part of the neural tube and, by doing so, define positioning of MHB along the anterior-posterior axis; Wnt1 and Fgf8 expression patterns are restricted to the mid-hindbrain junction in specular domains and are involved in the growth and maintenance of MHB cells; genes expressed across the entire mid- and hindbrain territory, such as Pax2, Pax5, En1/2, define the identity of this region, as a whole (Acampora *et al.* 1995, 1997; Suda *et al.* 1997). The second organizing center of the midbrain/hindbrain region is the FP. Sonic hedgehog (Shh), a secreted glycoprotein and the key-signaling molecule of the FP, is mainly supplied to the neural tube by the ventral midline structures. In mice lacking Shh (Matsunaga *et al.* 2000), the nervous system shows abnormalities in the development of ventral midline structures like floor plate, notochord and the differentiation of ventral cell types. Shh provided by the floor plate and notochord transforms the dorsal into ventral fates and is required for the ventral cell types differentiation (Alexandre & Wassef 2005). Midbrain dopamine neurons and 5-HT neurons are induced close to the floor plate around E10. Dopamine neurons appear rostral to the MHB, whereas 5-HT neurons are generated caudally. Dopamine neurons of the tegmentum respond to a combination of Shh and Fgf8 while serotonin neurons of the pons require early Fgf4 signalling, followed by Shh and Fgf8. However, Shh or Fgf8 or Fgf4 can not induce dopamine/serotonin neurons independently, indicating that an integration of dorsoventral and anteroposterior signals might be required (Ye *et al.* 1998).

Development of mDA progenitors.

The concomitant action of MHB and FP in the midbrain activates a combination of transcription factors including Otx2, Lmx1a/b, En1/2, Msx1/2, Ngn2 and Mash1, in a temporal sequence. The expression of Otx2, Lmx1b and En1/2 genes is already initiated

by E9.0 (Simeone *et al.* 1992; Ang *et al.* 1994; Simon *et al.* 2001; Smidt *et al.* 2000). Subsequently, Lmx1a and Msx1/2 expression turns on around E9.5, while Ngn2 and Mash1 are not expressed until E10.75 (Andersson *et al.* 2006a). The molecular mechanisms leading to the sequential activation of these genes is not understood. Shh can induce Lmx1a and Msx1/2 expression endogenously in mouse embryos one day later than the initiation of Shh expression (Echelard *et al.* 1993). These results suggest that Shh signalling induces another signal or factor that is required for the expression of Lmx1a and Msx1/2.

Induction of the mDA neurons

Birth dating studies demonstrate that mDA progenitors generate postmitotic immature mDA neurons between E9.5 and E13.5 in mice (Bayer *et al.* 1995). Immature mDA neurons induce Nurr1 expression (Zetterstrom *et al.* 1997) and En1/2 expression (Simon *et al.* 2001; Alberi *et al.* 2004) during this differentiation step (Figure 10). From E11.0 onwards, immature mDA neurons continue to migrate radially on radial glial fibres and further differentiating into mDA neurons (Kawano *et al*. 1995). These neurons express Pitx3, TH (tyrosine hydroxylase) and aromatic amino acid decarboxylase (Aadc, the enzyme that converts DOPA into dopamine), in addition to the earlier markers expressed in immature mDA neurons. Ngn2, however, is not expressed in mature mDA neurons. Aadc mRNA transcripts are thought to be expressed already in immature mDA neurons (Smidt *et al.* 2004).

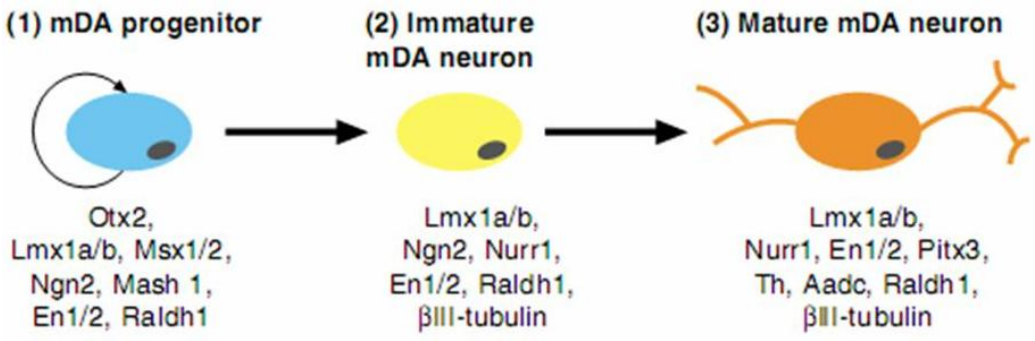


Figure 10. The sequential timing of transcription factor activation in mDA progenitors. The curved arrow indicates cycling cells. (Ang 2006)

Transcription factors required for mDA neuron development

The roles of transcription factors, such as Nurr1, En1/2 and Ptx3, acting during the late differentiation step of the mDA lineage (described briefly above, see Figure 10 and Table 1) have been extensively reviewed (Goridis & Rohrer 2002; Riddle & Pollock 2003; Wallen & Perlmann 2003; Simeone *et al*. 2005; Smits *et al.* 2006; Prakash *et al*., 2006). However, the roles of the transcription factors that govern the specification and early differentiation of mDA progenitors have only recently started to emerge during the past decade (Table 1).

Transcription factor	Expression in the mDA lineage	Function in mDA cells	References
Otx2	Progenitors	Required for regional and neuronal specification of mDA progenitors	Puelles et al., 2003; Puelles et al., 2004; Vernay et al., 2005
Msx1	Progenitors	Required for neuronal differentiation	Andersson et al., 2006b
Ngn2	Progenitors and immature neurons	Required for neuronal differentiation	Andersson et al., 2006a; Kele et al., 2006
Mash1	Progenitors	Not required for neuronal differentiation but can compensate for Ngn2 function	Kele et al., 2006
Lmx1b	Progenitors, immature and mature neurons	Required for maintenance of mature mDA neurons	Smidt et al., 2000
En1/2	Progenitors, immature and mature neurons	Required for the generation and survival of mature mDA neurons	Simon et al., 2001; Alberi et al., 2004
Nurr1	Immature and mature neurons	Required for the maintenance of mature mDA neurons and their expression of late differentiation markers	Zetterstrom et al., 1997; Saucedo-Cardenas et al., 1998; Wallen et al., 1999; Wallen et al., 2001; Smits et al., 2003
Pitx3	Mature neurons	Required for Th-expression in a subset of mature mDA neurons and for the survival of primary SNpc, and also VTA, neurons	Hwang et al., 2003; Nunes et al., 2003; van den Munckhof et al., 2003; Smidt et al., 2004; Maxwell et al., 2005

Table 1. A summary of the role of transcription factors in mDA neuron development (from Ang, 2006)

Otx2

Otx2 encodes a member of the bicoid sub-family of homeodomain-containing transcription factors that is widely expressed before gastrulation, but its expression becomes progressively restricted to the anterior third of the mouse embryo after E7.75 (Simeone *et al.* 1993; Ang *et al.* 1994). Within the nervous system, Otx2 expression is restricted to the forebrain and midbrain between E8.5 and E12.5. In addition to these anterior brain region, expression is also detected in the rhombencephalon from E12.5 onwards (Mallamaci *et al.* 1996). Otx2 is required for the formation of the forebrain and midbrain as a result of its role in the anterior visceral endoderm, where it functions to restrict posterior fates (Mallamaci *et al.* 1996; Perea-Gomez *et al*. 2001) (Simeone &

Acampora, 2001). Subsequently, Otx2 is also required for positioning the expression of Fgf8 and Wnt1 at the midbrain boundary (Brodski *et al.* 2003), and it limits the dorsal extent of Shh expression in the ventral midbrain (Puelles et al. 2003).

The homeobox-containing transcription factor Otx2 has additional roles in specification and differentiation of mDA progenitor (Simeone *et al.* 2002; Simeone 2005). At the midbrain/hindbrain boundary, Otx2 specifies identity and number of dopaminergic versus serotonergic progenitors by antagonizing the fibroblast growth factor 8 (Fgf8) and sonic hedgehog (Shh) 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 co-expressed with Engrailed 1 (*En1*) (Puelles *et al.* 2004).

Several conditional Otx2 mouse mutants have been generated to delete Otx2 at different developmental stages. For example, conditional mutant mice were generated to inactivate Otx2 by a Cre recombinase expressed under the control of the *En1* promoter ($En1^{cre/+}$; $Otx2^{flox/flox}$) (Figure 11). This strategy allowed to delete Otx2 only in the midbrain of $En1^{Cre/+}$; $Otx2^{flox/flox}$ embryos; from E9.5 (around 25 somites) onwards, a virtually complete inactivation of Otx2 was detected in the ventral and caudal midbrain and functional Otx2 transcripts were confirmed to the dorsolateral aspect of the anterior midbrain (Puelles *et al.* 2004).

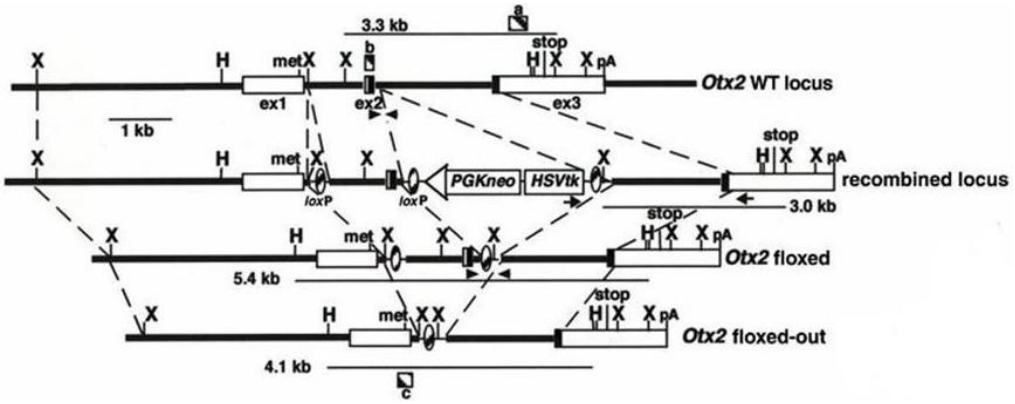


Figure 11. Wild-type locus (thick upper line) is compared to three Otx2 mutant loci: after homologous recombination with the targeting vector (second line), after removal of the PGK neocassette (third line) and after excision of the Otx2 exon 2 (fourth line). (Puelles et al., 2004)

In Otx2 conditional mutant mice (*En1*$^{cre/+}$*; Otx2*$^{flox/flox}$), midbrain dopaminergic neurons were greatly reduced in number and most of their precursors underwent neurotransmitter fate switch, generating 5-HT-positive neurons (Puelles *et al.* 2004) (Figure 12).

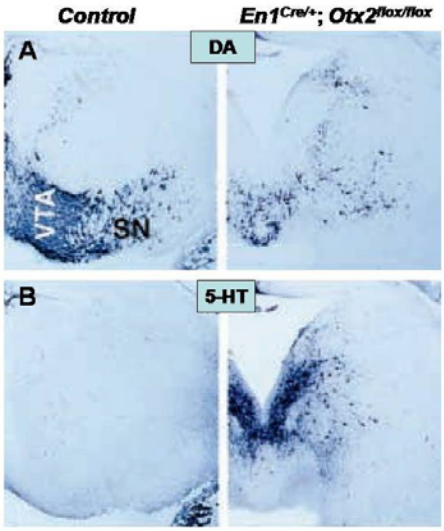


Figure 12. Abnormalities in the ventral midbrain of *En1*$^{Cre/+}$; *Otx2*$^{flox/flox}$ mutants. Th+ (A) and 5-HT (B) immunohistochemistry of DA and 5-HT area (Puelles *et al.,* 2004).

In these *En1*$^{Cre/+}$; *Otx2*$^{flox/flox}$ mutant embryos, midbrain expression of Shh expands dorsally, whereas Fgf8 expression, which is normally restricted to the anterior hindbrain, shifts anteriorly into the midbrain (Puelles *et al.* 2004). Despite these changes in AP and DV patterning molecules, a small domain of midbrain tissue develops normally. Within this domain, expression of the homeodomain protein Nkx2.2 expands ventrally into presumptive DA progenitors around E9.5, indicating that Otx2 is required for the repression of Nkx2.2 in these progenitors (Prakash *et al.* 2006). Interestingly, serotonergic neurons are generated ectopically in these Otx2 conditional mutants at the expense of TH+ mDA neurons. This alteration is maintained throughout life, since *En1*$^{cre/+}$*; Otx2*$^{flox/flox}$ adult mice still display reduced DA and increased 5-HT levels in the striatum and cerebral cortex (Borgkvist *et al.* 2006).

A different role for Otx2 in mDA progenitors was identified from studies of *Nestin-Cre*; *Otx2*$^{flox/flox}$ embryos (Vernay *et al.* 2005). In these conditional mutants, loss of Otx2 protein from E10.5 onwards results in loss of expression of the proneural genes Ngn2

and Mash1 in ventral mDA progenitors. Subsequently, mDA neurons are missing at the ventral midline of the midbrain. These results indicate that Otx2, presumably via regulating the expression of Ngn2 and Mash1, is also required for the generation of mDA neurons.

To study the role of Otx2 in the development of ventral midbrain dopaminergic neurons, mutant mice *En1*$^{Cre/+}$*; tOtx2*^{ov} was created (Omodei *et al.* 2008) in which Otx2 was conditionally over-expressed by a Cre recombinase under the control of the En1 promoter (Figure 13).

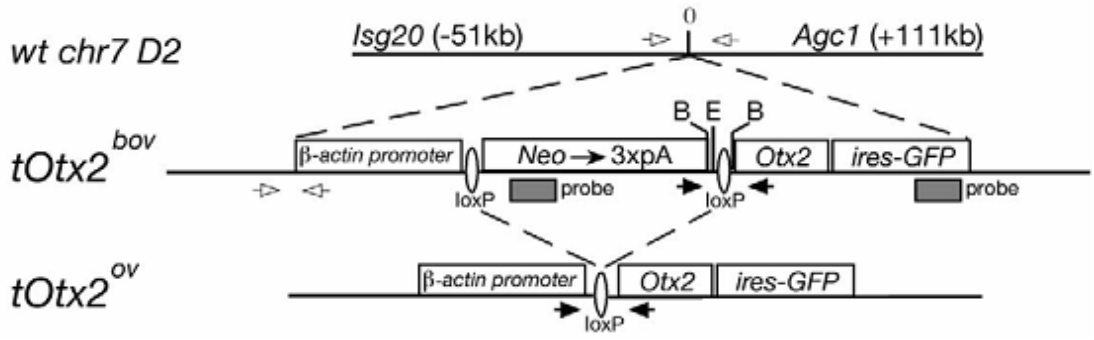


Figure 13. Generation of mouse mutants overexpressing Otx2. The genomic position at the chromosome 7 D2 region is shown in upper line whereas the tOtx2bov cassette is shown in second line, is inserted. Cre-mediated removal of the Neo-triple polyA stop cassette generates the tOtx2ov allele (third line). (from Omodei et al., 2008)

These mice over-express Otx2 gene at rostral midbrain and hindbrain. The comparison between the control mice *tOtx2*^{ov} and mutant mice *En1*$^{Cre/+}$*; tOtx2*^{ov} shows that mutant mice *En1*$^{Cre/+}$*;tOtx2*^{ov} in which Otx2 is overexpressed in hind brain region (prospective cerebellum) and over express throughout the midbrain. The Otx2 gene is essential for regulating the proliferation and differentiation of dopaminergic neurons. This over-expression of Otx2 gene in these mutant mice was linked to the position occupied by the midbrain dopaminergic progenitors which adjusting the number through a dose-dependent mechanism.

Indeed, *En1*$^{Cre/+}$*;tOtx2*^{ov} mice have an increase of 35% of dopaminergic progenitors neuron in the VTA of the anterior, and more prominently in posterior mesencephalon (Omodei *et al.* 2008) (Figure 14). Moreover, Otx2 controls selectively the development of dopaminergic neurons which modulates the number along the anterior-posterior axis of the ventral midbrain. Otx2 overexpression induces a selective expansion of both mesDA progenitors and neurons, without affecting identity and size of adjacent progenitor domains or their post-mitotic progeny. The features of the oculomotor (OM) and red nucleus (RN) was similar in *En1*$^{Cre/+}$*; tOtx2*^{ov} mice when compared with the *tOtx2*^{ov} mice (Omodei *et al.* 2008).

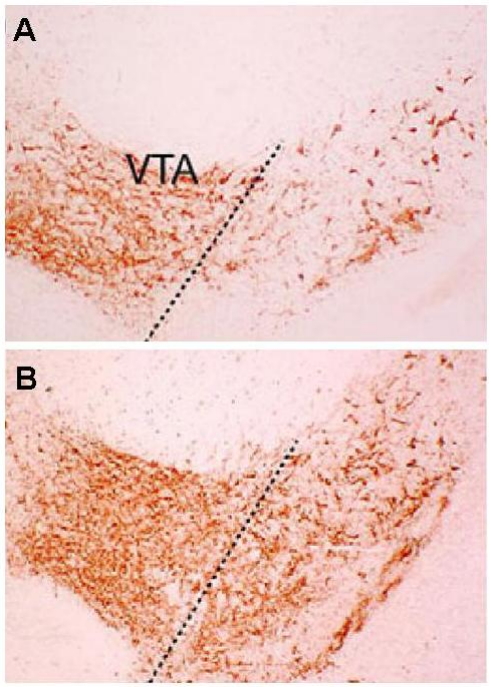


Figure 14. The overexpression of the Otx2 gene induces an increase in the number of dopaminergic neurons along the AP. Immnohistochemistry for the dopaminergic marker tyrosine hydroxylase (TH) at the level of the ventral midbrain clearly demonstrate that number of TH+ neuron were less in *tOtx2*^{ov} (A) in comparison to *En1*$^{Cre/+}$*;tOtx2*^{ov} (B) (Omodei *et al.* 2008).

Lmx1a and Lmx1b

Lmx1a and Lmx1b are members of the family of LIM homeodomain transcription factors. Lmx1a expression begins at E9.5 in the ventral midbrain and then progressively expands dorsally (Andersson *et al.* 2006). By contrast, Lmx1b is expressed in the midbrain from E8.0 onwards (Smidt *et al.* 2000), but this expression becomes restricted

by E9.5 to the mid-hindbrain boundary, roof plate and the ventral midbrain, including the floor plate. At E9.5, Lmx1b expression encompasses more cells in the ventral midbrain than does Lmx1a, but that by E10.5 the expression domains of the two genes largely coincide. Since the expression of Lmx1a directly overlies a region where TH+ neurons develop at E11.5, Lmx1a expression has been proposed to mark the dorsal boundary of mDA progenitors (Andersson *et al*. 2006).

Loss-of-function studies have shown that Lmx1b is required for the maintenance of TH+ mDA neurons. Recently, Lmx1a has been identified as a crucial determinant of mDA neuron fate development (Andersson *et al.* 2006). Overexpression of Lmx1a in the ventral midbrain promoted the generation of DA neurons over that of other neuronal subtypes. It is noteworthy that Lmx1a alone is not sufficient to induce mDA neurons, and that it functions cooperatively with ventral factors induced by the Shh pathway (Fig. 15).

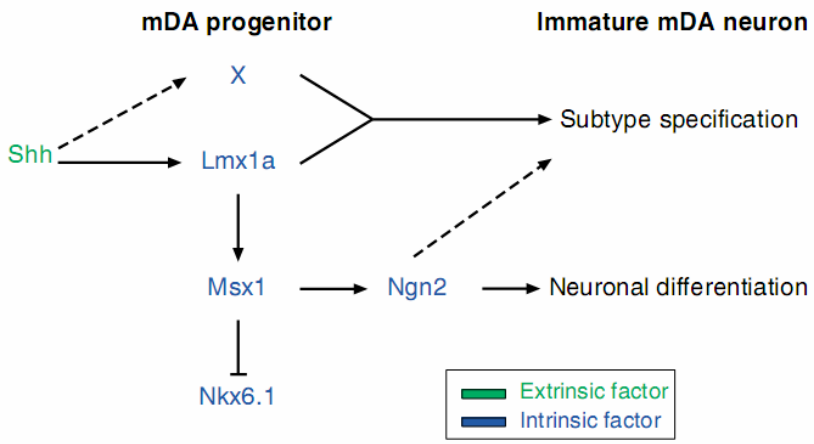


Figure 15. Model of mDA neuron specification. Shh induces Lmx1a and X (an unknown transcription factor) in mDA progenitors. Based on the timing of induction of endogenous Lmx1a expression compared with Shh expression, the induction of Lmx1a may be indirect. Lmx1a and X then act cooperatively to specify immature mDA neurons. Lmx1a in turn activates Msx1, which induces Ngn2. Ngn2 promotes neuronal differentiation and, perhaps, also the subtype specification of immature mDA neurons. In addition, Msx1 is required and is sufficient for the suppression of Nkx6.1 expression in DA progenitors. Dotted arrows indicate hypothetical functions that remain to be proven. This model is modified, from Andersson *et al*. 2006.

Additional support for cooperative interactions between Shh and Lmx1a has come from studies using the differentiation of embryonic stem (ES) cells. Mouse ES cells

transfected with Lmx1a differentiate into DA neurons in the presence, but not in the absence, of Shh (Andersson *et al.* 2006). Conversely, Lmx1a knockdown by siRNA electroporation resulted in a loss of DA neurons, which was not compensated for by unperturbed expression of Lmx1b (Andersson *et al.* 2006). This hypothesis is consistent with the observation that Lmx1b is much less efficient than Lmx1a at promoting mDA neuron differentiation in ES cells (Andersson *et al.* 2006).

Msx1 and Msx2

The mouse Msx genes, Msx1, Msx2 and Msx3, encode homeodomain transcription factors and function as transcriptional repressors (Ramos & Robert 2005). Msx1 and Msx2 are expressed in DA progenitors in the ventral midbrain (Andersson *et al.* 2006). Msx3, by contrast, is expressed exclusively in the dorsal aspect of the neural tube in the mouse, caudal to the mid-hindbrain boundary (Shimeld *et al.* 1996; Wang *et al.* 1996). Msx1-/- embryos exhibit a strong reduction in the normal number of mDA neurons, probably as a result of the downregulation of Ngn2 expression (Andersson *et al.* 2006). Moreover, Msx1 is required to repress Nkx6.1 expression in ventral midbrain progenitors (Andersson *et al.* 2006). Premature expression of Msx1 in the midbrain in transgenic mice also leads to the precocious expression of Ngn2 and Nurr1, and to the downregulation of Shh in the floor plate, indicating that Msx1 sets the timing of mDA neuron generation possibly by inducing Ngn2 expression in ventral midbrain progenitors (Andersson *et al.* 2006). Given that Msx genes normally function as repressors, Msx1 may regulate the activity of a repressor of Ngn2 in mDA progenitors.

Ngn2 and Mash1

Proneural genes Mash1, Ngn2 and Ngn1 show an intricate pattern of expression in the ventral midbrain. Ngn2 and Mash1 are expressed in mDA progenitors, whereas Ngn1, Ngn2 and Mash1 are co-localized in the ventricular zone more dorsally (Kele *et al*. 2006). Ngn2 is required for the generation of Nurr1+ immature mDA neurons, and probably also for their subsequent differentiation into TH+ mature mDA neurons (Andersson *et al.* 2006b; Kele *et al*. 2006). Although Mash1 by itself is not required for mDA neuron development, the loss of both Mash1 and Ngn2 in Mash1;Ngn2 double

mutant mouse embryos leads to a greater loss of mDA neurons than occurs in Ngn2 single mutants, suggesting that Mash1 can partially compensate for the loss of Ngn2 function in mDA progenitors. Accordingly, this results in a further rescue of Th+ neurons in Ngn2KIMash1/Mash1 embryos that express Mash1 under the control of the Ngn2 promoter (Kele *et al.* 2006). Ngn2 has a role in regulating generic neuronal, as well as subtype-specific, differentiation programs in other parts of the CNS (Bertrand *et al*. 2002). In other parts of the CNS, the role of Ngn2 in subtype specification has been demonstrated by the inability of other classes of proneural genes to compensate for Ngn2 activity (Bertrand *et al.* 2002). Mash1 is able to compensate partially for Ngn2 function, as 60% of the normal number of mDA neurons are generated in Ngn2KIMash1/KIMash1 embryos. This partial compensation suggests some unique role for Ngn2 in specification of the mDA neuronal subtype. In addition, the expression of Ngn2, but not Mash1, in postmitotic DA neurons is consistent with an additional and unique role for Ngn2 in regulating later differentiation steps in immature mDA neurons. However, Ngn2 alone is insufficient to promote the ectopic expression of DA neuron markers and the generation of ectopic DA neurons (Kele *et al*. 2006).

The Engrailed (En) homeobox genes are one of the most widely studied group of transcription factors, described and investigated in a variety of species. The En genes are involved in regionalisation during early embryogenesis (Hidalgo, 1996; Joyner, 1996), and later in the specification of certain neuronal populations (Lundell *et al.* 1996; Simon *et al.* 2001). During early embryogenesis, they are required for the maintenance of Fgf8 expression in the midbrain and hindbrain. During later development and throughout life, the two genes are required for the survival and maintenance of mesDA neurons in a cell-autonomous and gene dose-dependent manner. In mouse embryo expression of these genes was first detected at 8 day. In vertebrate species, two homologs of Engrailed exist, En1 and En2. At the protein level, the sequence differences between homologs and paralogs are significant, while homeobox domain is highly conserved. En1 has roles in generation of mid-hindbrain precursor cells and in signaling normal development of the limbs and sternum (Wurst 1994). Engrailed-1 is a target of Wnt-1 signaling pathway in the midbrain development. En1 mutant mice die at

birth with a large mid-hindbrain deletion, whereas En2 mutants are viable, with cerebellar defects. En1 mutant phenotype was rescued by replacement of En1 with En2 (Hanks *et al.* 1995). Engrailed-2 was ectopically expressed in cerebellar Purkinje cells from the late embryonic stage into adulthood (Baader *et al.* 1999).

The *En2* mutation was created by homologous recombination, resulting in the replacement of approximately 1 kb of the *En2* gene (300 bp of intron and 700 bp of the homeobox exon including the end of translation) (Figure 16; Joyner *et al.* 1991).

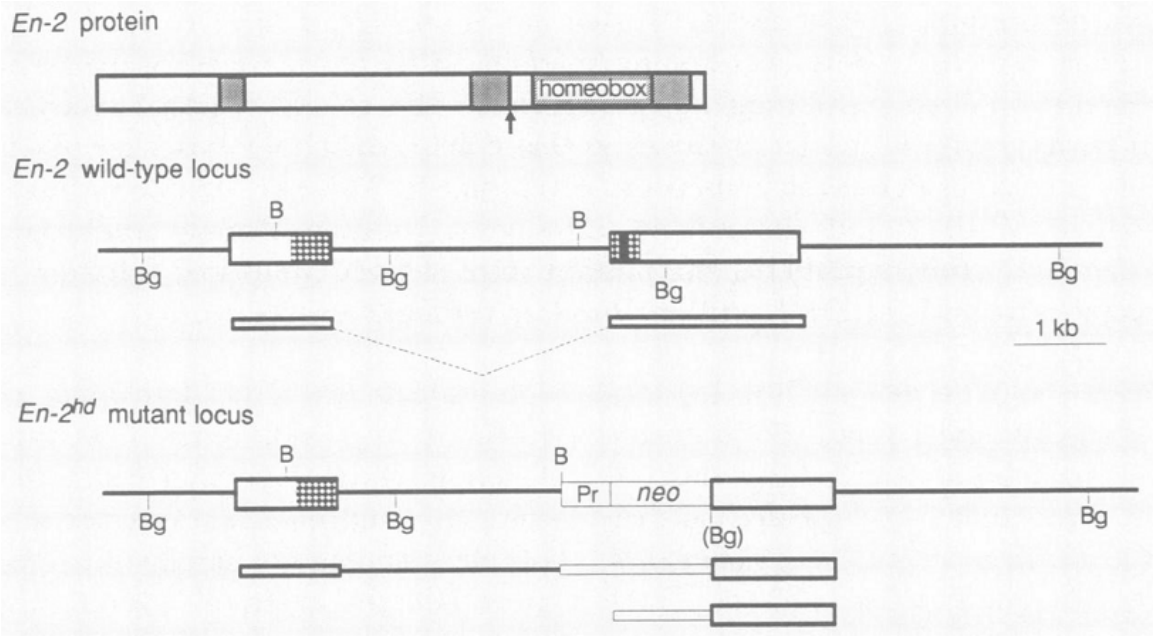


Figure 16. Structure of the En-2 protein and wild-type and mutant loci- The normal En-2 protein product is shown schematically at the top with the four engrailed conserved domains depicted as stippled boxes. The conserved domains NH2- and COOH-terminal of the homeobox are 17 and 21 amino acids, respectively. The arrow indicates the position of the intron in the En-2 gene. The En-2 wild-type (middle) and mutant (bottom) loci are shown schematically with the 5' end to the left. The En-2 exons are marked as thick-lined rectangles with the translated sequences stippled and the homeobox solid. The neo containing vector is shown as a thin-lined rectangle and Pr indicates the 500-bp human P-actin promoter sequences. The one transcript of the wild-type En-2 gene and two transcripts of the mutant En-2 locus from both the En-2 promoter and the 1-actin promoter are indicated below the loci with narrow rectangles indicating the exon sequences. The restriction sites are B, Barn HI and Bg, Bgl II. (Bg) indicates the Bgl II restriction site destroyed in making the mutation (from Joyner et al., 1991).

For generation of $En1^{+/-}/En2^{-/-}$ or $En1/tau\text{-}LacZ^{+/-}/En2^{-/-}$ mice, first *En1/tau-LacZ* mice were generated by a "knock-in" strategy in which the first 71 codons, including the start

codon, were replaced by a *tau-LacZ* sequence (Callahan and Thomas, 1994) and resulted in an En1 null allele. The construct and procedures are described below (Saueressig *et al.* 1999). Parental lines for producing the mutant mice deficient for both En1 and En2 were kept as $En1^{+/-}/En2^{-/-}$ or $En1/tau\text{-}LacZ^{+/-}/En2^{-/-}$ (Simon *et al*. 2001) (Figure 17)

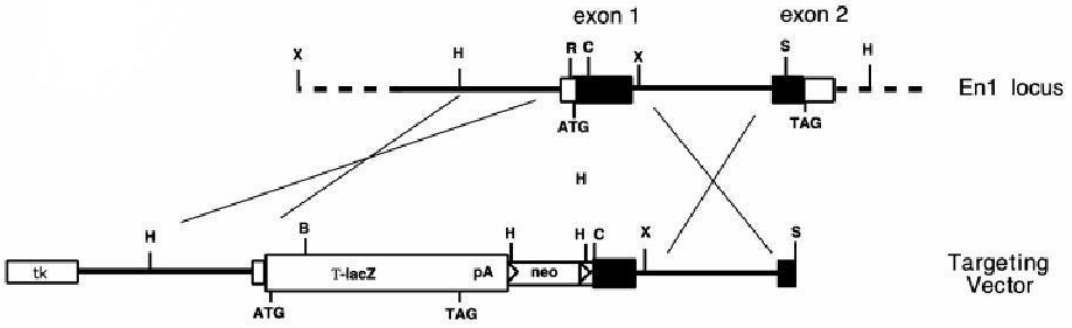


Figure 17. Structure of the mouse En1 locus- The *En1/tau-LacZ* knock-in targeting vector is shown below. Abbreviations: B, *Bam*HI; C, *Cla*I; H, *Hind*III; R, *Eco*RI; X, *Xba*I; neo, PGKneopA G418 resistance cassette used for positive selection flanked by loxP recombination sites (triangles); T-lacZ, coding region of the tau-lacZ fusion gene; pA, SV40 polyadenylation signal. DNA probe: A 0.7 kb *Eco*R1-*Hind*III fragment from the 3′ end of the *En1* gene was used to screen ES cells for homologous recombinants (Saueressig *et al.* 1999).

The single-null mutants for either En1 ($En1^{-/-}$) or En2 ($En2^{-/-}$) show no significant alterations in the organization of the mesDA system at birth. $En1^{-/-}$, $En1^{-/-}; En2^{+/-}$ and $En1^{-/-}; En2^{-/-}$ mice die at birth and show a gene-dose- dependent reduction of mesDA neurons ((Simon *et al*. 2001). Mice of other Engrailed genotypes are viable and fertile. Among these, $En1^{+/-}$, $En1^{+/-}/En2^{+/-}$, $En2^{+/-}$, and $En2^{-/-}$ mice displayed a wild-type-like distribution of the neurons at all ages. $En1^{+/-}; En2^{-/-}$ (En^{HT}) mice are viable and fertile and showed a specific loss of DA neurons in the SN.

In the En^{HT} mice, the numbers of mesDA neurons continued to decrease until 3 months after birth while the distribution and number of mesDA neurons were stablized in $En2^{-/-}$ mice at all age. Indeed, from 3 months after birth, the mutants had on average 32.6% fewer mesDA neurons than their $En2^{-/-}$ litter-mate controls (Sgado *et al*. 2006) (figure 18). Moreover, no major defect in DA and 5-HT systems has been found in the $En2^{-/-}$ mice so for this purpose $En2^{-/-}$ mice has been considered as control littermates (Sgado *et al*. 2006). In the open field test, a general assessment of locomotor and exploratory

behavior, En^{HT} mice were not impaired at 8 months; however, by 18 months of age, they showed a significant reduction in forward locomotion compared with $En2^{-/-}$ littermate controls and to their own performance at 8 months, whereas in $En2^{-/-}$ mice, locomotion was not significantly altered (Sgado *et al*. 2006).

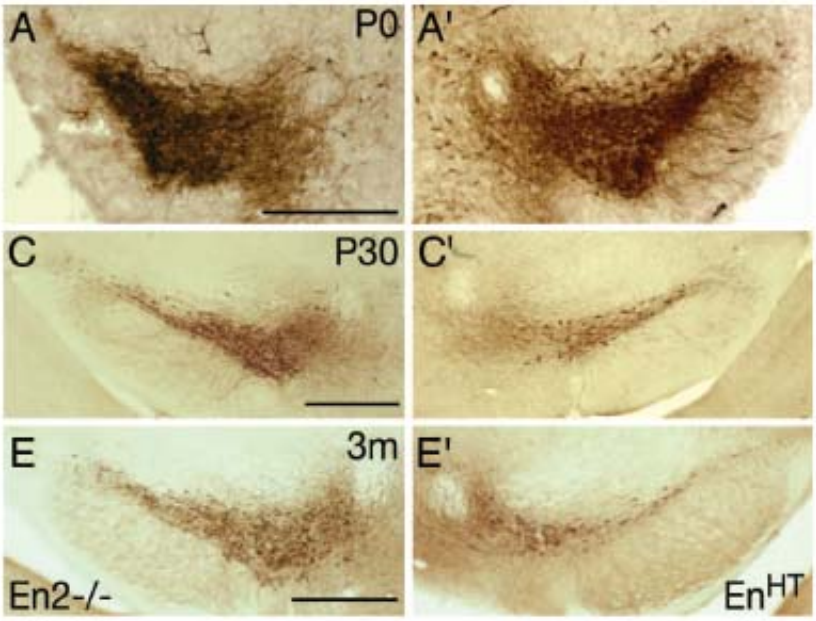


Figure 18. Progressive postnatal degeneration of dopaminergic cells of the substantia nigra-TH immunostaining on coronal brain section at P0 (A, A'), P30 (C, C'), and 3months (E, E') of $En2^{-/-}$and En^{HT} mutant mice on the level of the substantia nigra. (Scale bars: 0.5mm) (from Sgadò *et al.* 2006).

Development of hind brain serotonergic neurons.

Genetic and transplantation experiments have demonstrated that the sonic hedgehog (Shh) signal, which is emitted by the notochord and the floorplate (midline of the neural tube), is required to induce 5-HT cell fate. Although 5-HT neurons are born near the floorplate, they migrate to specific positions along the dorso-ventral axis as the raphe nuclei are formed (refs?).

Induction of the hindbrain 5-HT neurons

The inductive requirements of 5-HT neurons of the DRN (dorsal raphe nucleus) differ from those of the MRN (medial raphe nucleus). The first 5-HT neurons that are born in r1 (rhombomere 1) become the 5-HT neurons of the DRN, and may be particularly

dependent on Fgf8 or another signal from the mid/hindbrain organizer, also known as the isthmus. When Fgf8 expression is reduced by shifting expression of the homeodomain gene Otx2 caudally, 5-HT neurons in the DRN are reduced (Brodski 2003). Conversely, if the expression of the Gbx2 homeodomain gene is shifted rostrally and Fgf8 expression extended, the DRN is enlarged (Wassarman *et al.* 1997). The 5-HT neurons of the DRN also rely on a non-cell autonomous, dosage-sensitive function of the En1 and En2 transcription factors, which again could be mediated by altered Fgf8 expression (Cordes 2005). Taken together, these data appear to suggest that high early levels of Fgf8 or another isthmus-specific signal may be required to induce 5-HT neurons in the DRN, while lower Fgf8 levels may suffice to induce 5-HT neurons in the MRN.

Transcriptional determinants of early 5-HT neuron specification

The transcription factors involved in 5-HT neuron development can be roughly divided into two broad classes:

1) Nkx2.2, Nkx6.1, and Mash1 are required to generate 5-HT precursors,
2) Mash1, Gata2, Gata3, Lmx1b, and Pet1 are required for -5-HT subtype selection and 5-HT neuron terminal differentiation.

Nkx2.2 homeodomain transcription factor acts downstream of Shh signaling. Nkx2.2 is essential for initiating the specification of all 5-HT neurons in the raphe except for those from the DRN. In Nkx2.2–/– mice, only dorsal raphe 5-HT neurons are present, and all others are missing. Nkx2.2 is thought to promote 5-HT neuron differentiation in part by down regulating the homeodomain transcription factor Phox2b. During 5-HT neuron development, Nkx2.2 does collaborate with the related homeodomain transcription factor Nkx6.1, which is expressed more broadly than Nkx2.2 in the ventral hindbrain. Nkx2.2 and Nkx6.1 together direct Gata2 and Gata3 expression and 5-HT neuron specification. As already described, Nkx2.2 expression is negatively controlled by Otx2 in presumptive DA progenitors. De-repression of Nkx2.2 in these progenitors due to conditional knockout of Otx2 in these cells results in ectopic generation of serotonergic neurons in place of mDA neurons (Puelles *et al.* 2003, 2004).

Nkx6.1 is a Shh-inducible HD transcription factor similar to Nkx2.2 but with a broader expression domain (Briscoe *et al.* 2000). Shh regulates Gata2 and 5-HT expression in r1 through a combination of Nkx2.2 and high Nkx6.1 signals (Figure 19). Down-regulation of Nkx6.1 by antisense morpholinos in the hindbrain leads to loss of Gata2, Gata3, and Pet1 expression and the absence of 5-HT neurons (Craven *et al.* 2004). The Gata2 and Gata3 zinc finger transcription factors are required in a global 5-HT neuron-specific and a 5-HT cluster-specific manner, respectively (Cordes *et al.* 2005) (Figure 19). Ectopic expression of either Gata protein is sufficient to induce 5-HT neurons in r1 of the hindbrain, and this occurs downstream of Nkx2.2/Nkx6.1, and upstream of Lmx1b /Pet1 (Craven *et al.* 2004). Gata3 is expressed in both clusters of 5-HT neurons starting at E10.5–11.5, but it is only required for development of 5-HT neurons in the caudal raphe nuclei (Pattyn *et al.* 2004). In Gata3–/– embryos, a normal number of 5-HT precursor cells are formed, but a striking gradient of diminishing requirement for Gata3 from caudal to rostral can be seen (Pattyn *et al.* 2004).

Figure 19. The specification of rostral 5-HT neurons in the vertebrate hindbrain. Shh signaling in the ventral midline activates the Nkx2.2 and Nkx6.1 in the rostral hindbrain. Nkx2.2 and Nkx6.1 are sufficient to activate expression of Gata2 and Gata3, which can positively regulate each other. Gata2, in turn, is necessary and sufficient to activate Lmx1b and Pet1, and to specify 5-HT neurons. Gata2 may activate additional transcription factors and/or may be required to directly cooperate with Lmx1b and Pet1 in 5-HT specification (modified from Cordes 2005)

Two lines of evidence suggest that terminal differentiation of 5-HT neurons depends on Lmx1b. First, no expression of differentiated 5-HT neuronal markers such as 5-HT, serotonin transporter (SERT) and Pet1 are detected in the Lmx1b-/- mutant mice. Second, most Lmx1b–/– cells exhibit aberrant migratory behavior at a late stage of their development. Lmx1b-positive 5-HT precursors were derived from Nkx2-2-expressing precursors. Lmx1b-mediated event provides a critical step that couples the Nkx2-2-

dependent early specification of neurons with the Pet1-dependent terminal differentiation of 5-HT neurons (Ding *et al.* 2003). Pet1 working synergistically with GATA3 in the caudal raphe nuclei and other unidentified Pet1-independent transcription factors that are downstream to Lmx1b, control the terminal differentiation of 5-HT neuron (Ding *et al.* 2003).

AIM OF THESIS

The aim of this thesis was to investigate whether an altered embryonic development of dopaminergic and serotonergic neurons could alter seizure susceptibility in the adult life. To this purpose, a series of classical and conditional knockout mouse lines with targeted inactivation of Otx2 and En genes were studied. The mouse lines used in this study are as follow.

1) $En1^{cre/+}$; $Otx2^{flox/flox}$ conditional mutant mice in which midbrain dopaminergic neurons were greatly reduced and 5-HT positive neurons increased.
2) $En1^{Cre/+}$;$tOtx2^{ov}$ condition mutant mice in which dopaminergic neurons were greatly increased.
3) $En1^{+/-}$; $En2^{-/-}$(En^{HT}) mice which have progressive postnatal degeneration of dopaminergic cells.

Using these mouse lines, we addressed how an altered development of dopamine and serotonin neurotransmitter pathways can markedly affect seizure susceptibility in the adult brain. As a seizure model, systemic administration of the glutamate agonist kainic acid (KA) was used. Behavioural observation of KA-induced seizures was performed, and induction of immediate early genes (IEGs) like *c-fos* and *c-jun* was also followed after KA seizures. In order to assess whether altered susceptibility to KA-induced seizure in these mice also resulted in altered susceptibility to long-term damage, histological and immunohistochemical stainings were also performed.

MATERIALS AND METHODS

Animals.

The generation and genotyping of *En1*$^{Cre/+}$; *Otx2*$^{flox/flox}$ (Puelles et al., 2003; 2004), *En1*$^{Cre/+}$*;tOtx2*^{ov} (Omodei et al., 2008), *En*^{HT} (Sgadò et al., 2006) and *En2*$^{-/-}$ (Joyner et al., 1991) mutant mice have been already reported.The two strains (*En1*$^{Cre/+}$ x*Otx2*$^{flox/+}$ and *En1*$^{Cre/+}$ x*tOtx2*^{ov}) were mated to generate parental mice (*En1*$^{Cre/+}$; *Otx2*$^{flox/+}$,*Otx2*$^{flox/flox}$, *En1*$^{Cre/+}$*;tOtx2*^{ov} and *tOtx2*^{ov}).*Otx2*$^{flox/flox}$ and *tOtx2*^{ov}mice were chosen as controls, as they do not show any anatomical or behavioral abnormality respect to wild-type animals (Puelles *et al.* 2003; Borgkvist *et al.* 2006, Omodei *et al.* 2008). Adult (3-6 months old; weight = 20-35 g) mice of both sexes were used. The *En*^{HT} and *En2* mutants (mixed 129Sv x Swiss–Webster genetic background) were crossed at least three times into a C57BL/6 background. Adult (5 months old; weight = 25-35 g) male mice were used in all experiments. Animals were housed in a 12 hr light/dark cycle with food and water available *ad libitum*. Experiments were conducted in conformity with the European Communities Council Directive of 24 November 1986 (86/609/EEC). Additional details on the mouse strains used in our studies can be found in Tripathi *et al.*, 2008, 2009.

Drug treatments.

For seizure studies, *Otx2*$^{flox/flox}$ (n= 10), *En1*$^{Cre/+}$; *Otx2*$^{flox/flox}$ (n= 10), *En1*$^{Cre/+}$*;tOtx2*^{ov} (n= 7), *tOtx2*^{ov} (n=7), *tOtx2*^{ov} (n=7), *En*^{HT} (n=8), Wild type (n=8), *En2*$^{-/-}$ (n= 12) mice received a single intraperitoneal injection of kainic acid (KA; Ocean Produce International, Shelburne, NS, Canada; dissolved in saline) at 20 mg/kg.For pCPA+KA treatments, mice (n = 10 per genotype), received the same dose of KA 16 hr after the last pCPA injection. All experiments were performed blind to genotype and treatment.
To deplete 5-HT, Otx2 conditional mutant mice and their controls (n = 5 per genotype) received *pCPA* (4-chloro-L-phenylalanine hydrochloride, Sigma; 10 mg/ml stock in saline) twice a day (≈ 10:00 and 18:00 hr) at a dose of 100 mg/kg (i.p.) 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 hr after the last *pCPA*/saline injection, brains were dissectedOne hemisphere was used for immunohistochemistry, and the other for HPLC.

Behavioural observation of 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 hr 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.

5-HT dosage by HPLC.

5-HT was measured according to Atkinson *et al.* (2006) in the *Otx2*$^{flox/flox}$ and *En1*$^{Cre/+}$; *Otx2*$^{flox/flox}$ mice. Brain areas (pons/ventral midbrain, hippocampus and cerebral cortex) were dissected on ice, weighed to the mg sensitivity and extracted with a buffer containing 8.2% ascorbic acid, 1.64% $Na_2S_2O_5$, 0.83M $HClO_4$. Extraction buffer volume (in μl) corresponded to three times the weight in mg 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) in extraction buffer. Twenty μl of samples or standards were injected into a Synergy Hydro-RP separation column, fitted with a C18 cartridge column (Phenomenex, Bologna, Italy). 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 Millenium software. Values (± SE) were reported as pmol of 5-HT per mg of wet tissue. Statistical analysis was performed by one-way ANOVA followed by post-hoc Tukey test.

In situ hybridization.

In situ hybridization experiments were performed to detect IEGs activation following KA seizures. Mice were killed at 2 hr (for $En1^{Cre/+}$; $Otx2^{flox/flox}$) or 3 hr (for $En1^{Cre/+}$; $tOtx2^{ov}$, $tOtx2^{ov}$, En^{HT} and $En2^{-/-}$) 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, taken at the level of the dorsal hippocampus, were analysed using the Image J free software (http://rsb.info.nih.gov/ij/). For each section, signal intensity was measured in ten different circular windows (area = 0.01 mm^2) placed in layers 2-3 and 5-6 of the parietal/temporal cortex. Mean signal intensity was divided by the background labeling calculated in the corpus callosum. Statistical analysis was performed by Student's t-test.

Immunohistochemistry.

Brains were fixed by immersion in 4% paraformaldehyde, cryoprotected in 30% sucrose/1xPBS and coronal sections (40 μm thick) were cut on a freezing microtome. Serial sections were incubated overnight with different antibodies (anti-5-HT, anti-SERT, anti-NeuN, anti-NPY, anti-Parvalbumin, anti-Somatostatin, see the table 2 for dilution), 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,

Burlingame, CA) followed by avidin-biotin-peroxidase complex (ABC kit, Vector Laboratories) and diaminobenzidine reaction.

Quantitative analyses of 5-HT immuhistochemistry experiments in control and Otx2 mutant mice were performed on digitized images (10x primary magnification) by using the Metamorph software. Three sections at the level of the ventral tegmental area (VTA), CA3 subfield (dorsal hippocampus) and dorsal raphe nucleus were taken from each animal (3-5 animals per genotype). To count 5-HT positive cells in the VTA, four squared counting boxes (75 mμ per side) were taken per section. 5-HT staining in the CA3 pyramidal layer was measured in 20 sampling windows (approximately the size of one cell) per section, and obtained values (grey levels) were divided by the background value measured in the callosum. 5-HT staining per cell in the dorsal raphe nucleus was measured in 10 cell bodies per section, and obtained values (grey levels) were divided by the background value measured in the callosum. Statistical analyses were performed by one-way ANOVA followed by appropriate post-hoc test

Neuronal damage in WT and *En2*$^{-/-}$ mice was qualitatively in CA1/CA3 areas according to the following scale (Bozzi *et al.* 2000; Bozzi and Borrelli, 2002; Cilio *et al.* 2001): little damage, presence of scattered degenerated cells; mild damage, small areas with degenerated cells and/or tissue sclerosis; severe damage, extended areas of neuronal and fiber degeneration, accompanied by tissue sclerosis. Neurodegeneration was also confirmed by Nissl staining, performed on sections adjacent to those used for NeuN histochemistry.

Quantitative analyses of PV, SOM and NPY-positive cells in WT *En2*$^{-/-}$ mice were performed on digitized images (20x primary magnification) by using the Metamorph software. Three sections at the level of the dorsal hippocampus and overlying somatosensory cortex were taken from each animal (3-5 animals per genotype). To count positive cells in cortical layers 2-3 and 5-6, six squared counting boxes (100 mμ per side) were taken per section. To count positive cells in the hilus, all cells were counted within each hilus, and values were expressed as the number of cells per hilar area. Statistical analyses were performed by one-way ANOVA followed by appropriate post-hoc test

Antibody	Company/Species/Serotype	Dilution
5-HT	Sigma-Aldrich, rabbit, polyclonal	1:5000
5-HT	Millipore, rat/monoclonal	1:200
SERT	Calbiochem, rabbit, polyclonal	1:5000
NeuN	Chemicon, mouse, monoclonal	1:500
NPY	Bachem, rabbit, polyclonal	1:5000
Parvalbumin	Sigma –Aldrich, mouse, monoclonal	1:5000
Somatostatin	Bachem, rabbit, polyclonal	1:5000

Table 2. Antibodies used during the immunohistochemistry experiments and their respective dilution.

Quantitative RT-PCR for *En2* mRNA

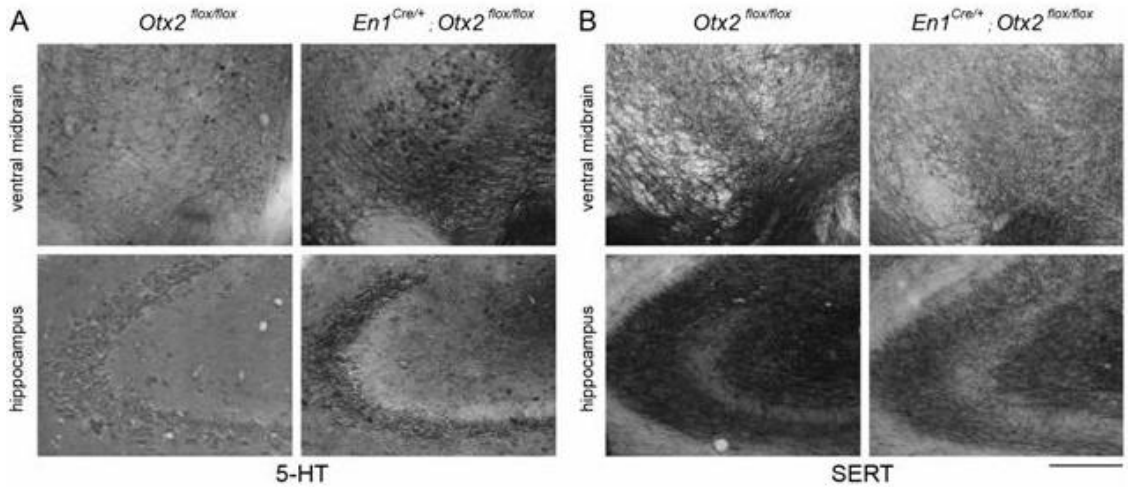
Total RNAs were extracted by Trizol© reagent (Invitrogen) from the cerebral cortex, hippocampus, ventral midbrain and cerebellum of four adult WT mice and pooled. DNAse-treated RNAs were purified and concentrated with Nucleospin RNA XS columns (Macherey-Nagel). cDNA for real-time PCR was synthesized from RNA (2 μg) using the Reverse Transcriptase Core kit (Eurogentec) 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 (Sigma Genosys, UK) were as follows: *En2* forward 5'-AGAGAGGGCGCAGTTCTTTG-3'; *En2* reverse 5'-GACACAGACGCAGACACAC-3' (GenBank accession no. NM_010134.3; expected fragment size: 151 base pairs); L41 forward 5'-GGTTCTCCCTTTCTCCCTTG-3'; L41 reverse 5'-GCACCCCGACTCTT-AGTGAA-3' (GenBank accession no. NM_001031808.2; expected fragment size: 179 base pairs). Each PCR cycle consisted of denaturation for 10 s at 94 ºC, annealing for 20 s at 60 ºC (58 ºC for L41), and extension for 30 s at 72 ºC. The fluorescence intensity

of SYBR green I was read and acquired at 72 ºC after completion of the extension step of each cycle. PCR conditions for individual primer sets were optimised 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). 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.

En1$^{Cre/+}$; *Otx2*$^{flox/flox}$ mice

Distribution of 5-HT and 5-HT transporter (SERT) in the ventral midbrain and hippocampus

We first analyzed the distribution of 5-HT and 5-HT transporter (SERT) in the ventral midbrain and hippocampus of drug-free *En1*$^{Cre/+}$; *Otx2*$^{flox/flox}$ mutant and *Otx2*$^{flox/flox}$ control adult mice.



C

Mice	*5-HT cells in VTA* *(cells/area)*	*5-HT staining in the CA3* *(staining density/cell)*
Otx2$^{flox/flox}$ mice	7 ± 1	1.155
En1$^{Cre/+}$; *Otx2*$^{flox/flox}$ mice	14 ± 3	1.491

Figure 20. 5-HT is increased and SERT is decreased in the ventral midbrain and hippocampus of *Otx2* conditional mutant mice. Figures 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. Quantitative analysis confirmed the increased number of 5-HT cells in the VTA and 5-HT staining in the CA3 area of *En1*$^{Cre/+}$; *Otx2*$^{flox/flox}$ mice (C). Scale bar = 150 μm.

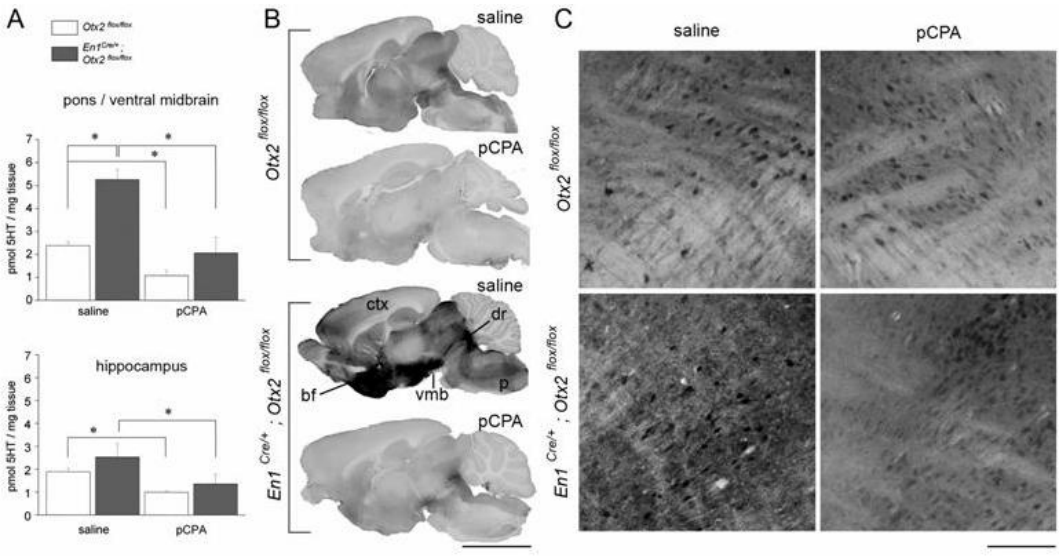
Immunohistochemistry experiments confirmed the presence of 5-HT-positive neurons in the ventral midbrain of *En1*$^{Cre/+}$; *Otx2*$^{flox/flox}$ but not *Otx2*$^{flox/flox}$ mice (Figure 20A). In

the hippocampus, 5-HT staining was increased in the CA3 area of *Otx2* conditional mutant mice, as compared to control mice (Figure 20A). These findings were confirmed by quantitative analysis. A small number of 5-HT positive cells were detected in the ventral tegmental area (VTA) of $Otx2^{flox/flox}$ mice. These cells had a very small size and a rounded shape, suggesting that they were likely platelets (Brenner *et al.* 2007). Conversely, a higher number of 5-HT positive cells, clearly identifiable as neurons, was detected in the VTA of $En1^{Cre/+}$; $Otx2^{flox/flox}$ mice. Quantitative analysis confirmed the increased number of 5-HT cells in mutant mice (mean number of cells per counting box, ± SE: control, 7 ± 1; mutant, 14 ± 3; n = 36 counting boxes from 3 mice per genotype; t-test, $p<0.05$) (Figure 20C). Quantitative analysis also confirmed increased 5-HT staining in the CA3 area of *Otx2* conditional mutant mice, as compared to control mice (median value of 5-HT staining / background: control, 1.155; mutant, 1.491; n = 180 sampling windows from 3 mice per genotype; Mann-Whitney rank sum test, $p<0.001$) (Figure 20C). Conversely, SERT levels in serotonergic fibers were markedly reduced in these areas in $En1^{Cre/+}$; $Otx2^{flox/flox}$ mice, when compared to control animals (Figure 20B).

5-HT levels in ventral midbrain and hippocampus

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, as compared to control mice (Figure 21A, saline-treated groups; one-way ANOVA, $p<0.05$; post-hoc Tukey test control vs. mutant, $p<0.05$). A slight but not significant increase of 5-HT content was detected in the whole hippocampus of $En1^{Cre/+}$; $Otx2^{flox/flox}$, as compared to $Otx2^{flox/flox}$ mice (one-way ANOVA, $p>0.05$). According to a previous study (Borgkvist *et al.* 2006), 5-HT levels were also increased in the cerebral cortex of mutant mice (pmol 5-HT/ mg 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 test, $p<0.05$). In both control and *Otx2* conditional mutant mice (n = 5 per genotype), prolonged treatment with the 5-HT synthesis inhibitor para-chlorophenylalanine (*pCPA*) significantly reduced 5-HT levels in the pons, ventral

midbrain and hippocampus (Figure 21A). *pCPA* also decreased 5-HT content in the cerebral cortex in both genotypes (pmol 5-HT/ mg 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 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 (Figure 21A).



D

Mice	*Treatment*	*5-HT staining per cell in the DRN (arbitrary units)*
Otx2$^{flox/flox}$	Saline	1.90±0.02
En1$^{Cre/+}$; *Otx2*$^{flox/flox}$	Saline	2.22±0.02
Otx2$^{flox/flox}$	*pCPA*	1.65±0.03
En1$^{Cre/+}$; *Otx2*$^{flox/flox}$	*pCPA*	1.63±0.03

Figure 21. 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, following a 3 days treatment with saline or pCPA. Data are reported as mean ± SE (n = 5 animals per group). *, $p<0.05$, post-hoc Tukey 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; ctx, 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. D) Quantitative analysis shows Mean values (± SE) of 5-HT staining per cell (normalized to background) in the dorsal raphe nucleus of *En1*$^{Cre/+}$; *Otx2*$^{flox/flox}$ and *Otx2*$^{flox/flox}$ mice, treated with saline or pCPA. Genotypes and treatments are as indicated. Scale bar = 150 μm.

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 (Figure 21A, B and Borgkvist *et al.* 2006), increased 5-HT staining was detected in several areas including ventral midbrain, basal forebrain, cerebral cortex (Figure 21B) and pons (raphe nuclei, Figure 21B, C) of saline-treated *En1*$^{Cre/+}$; *Otx2*$^{flox/flox}$ mutants, as compared to saline-treated *Otx2*$^{flox/flox}$ controls. Treatment with pCPA markedly reduced 5-HT staining in all these areas in both genotypes (Figure 21B, C, D). Quantitative analysis of 5-HT staining per cell in the dorsal raphe nucleus confirmed these findings (Figure 21D). Mean values (± SE) of 5-HT staining per cell (normalized to background) were as follows: Otx2$^{flox/flox}$ saline, 1.90±0.02; En1$^{Cre/+}$; Otx2$^{flox/flox}$ saline, 2.22±0.02; Otx2$^{flox/flox}$ + pCPA, 1.65±0.03; En1Cre/+; Otx2$^{flox/flox}$ + pCPA, 1.63±0.03 (n = 150 cells from 5 animals per group) (Figure 21D). Statistical analysis confirmed that 5-HT levels were increased in saline-treated mutant mice, as compared to controls (one-way ANOVA, $p<0.001$; post hoc Holm-Sidak test, $p<0.001$) and that pCPA significantly decreased 5-HT staining in both genotypes (one-way ANOVA, $p<0.001$; post hoc Holm-Sidak test, $p<0.001$, control saline vs. control pCPA, and mutant saline vs. mutant pCPA). No difference was detected between control and mutant mice treated with pCPA (one-way ANOVA, $p>0.05$; post hoc Holm-Sidak test, $p>0.05$).

5-HT levels alter seizure susceptibility

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 hr. 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 (Figure 22). The mean latency to the first generalized seizure in KA-treated *Otx2*$^{flox/flox}$ mice was 18.9 ± 8.7 min (Table 3). Progression of clinical signs was dramatically different in *En1*$^{Cre/+}$; *Otx2*$^{flox/flox}$ animals (Figure 22). Indeed, the trajectory in behavior score of *En1*$^{Cre/+}$; *Otx2*$^{flox/flox}$ mice differed from that of control mice starting from 20 min following KA administration (two-way repeated

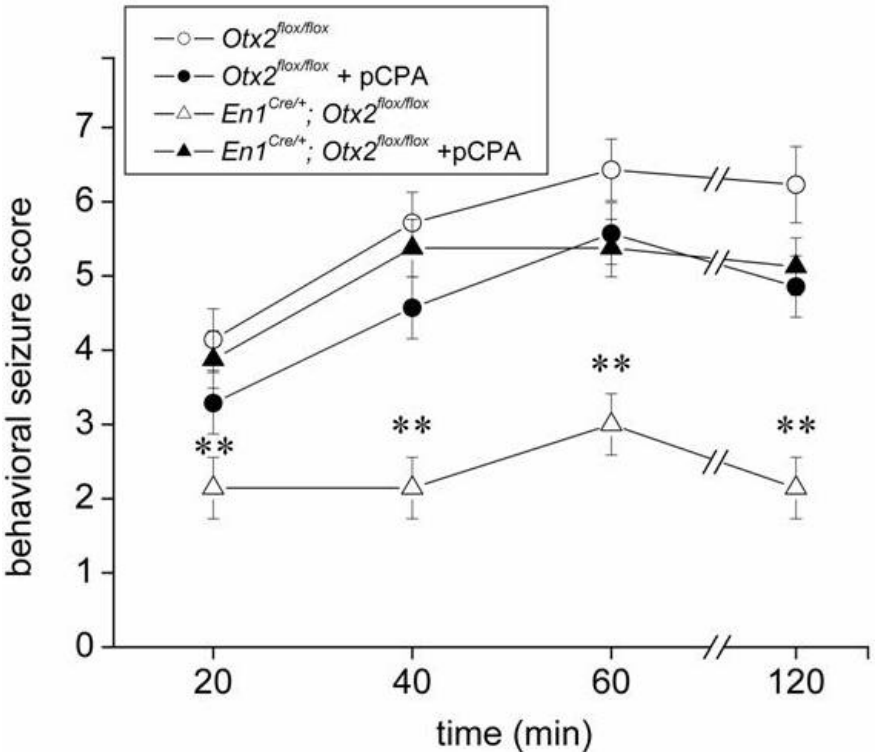


Figure 22. Resistance to KA-induced seizures in *Otx2* conditional mutant mice is abolished by 5-HT depletion. Graph shows the progression of behavioral changes over a 2 hr observation period following KA in control and *Otx2* conditional mutant mice, with or without pCPA pre-treatment. Genotypes and treatments are as indicated. Data are reported as mean seizure scores ± SE (n = 10 animals per group). **, $p < 0.001$, post hoc Holm-Sidak test, $En1^{Cre/+}$; $Otx2^{flox/flox}$ vs. the other three treatment groups.

measures ANOVA, $p < 0.001$; post hoc Holm-Sidak test, $En1^{Cre/+}$; $Otx2^{flox/flox}$ vs. $Otx2^{flox/flox}$ mice, $p < 0.001$). The majority of *Otx2* conditional mutant mice displayed only pre-convulsive behaviors, never showing any sign of generalized seizure activity (Figure 22). Only 1 out 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 3). $En1^{Cre/+}$; $Otx2^{flox/flox}$ never showed any sign of generalized seizure activity also at later times (> 2 hours) after KA administration (data not shown). Depletion of endogenous 5-HT by pre-treatment 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 (Figure 22). Indeed, seizure severity in $En1^{Cre/+}$; $Otx2^{flox/flox}$ pre-treated with *pCPA* was significantly

different than that observed in the same mice without *pCPA* (two-way repeated measures ANOVA, $p < 0.001$; post hoc Holm-Sidak test, *En1*$^{Cre/+}$; *Otx2*$^{flox/flox}$ + *pCPA* vs. *En1*$^{Cre/+}$; *Otx2*$^{flox/flox}$, $p < 0.001$; *En1*$^{Cre/+}$; *Otx2*$^{flox/flox}$ + *pCPA* vs. *Otx2*$^{flox/flox}$, $p > 0.05$).

Latency to first generalized seizure in *Otx2* conditional mutant mice pre-treated with *pCPA* was comparable to that observed in *Otx2*$^{flox/flox}$ mice (Table 3). Pre-treatment 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 pre-treated with *pCPA* (two-way repeated measures ANOVA, $p>0.05$; post hoc Holm-Sidak test, *Otx2*$^{flox/flox}$ + *pCPA* vs. *Otx2*$^{flox/flox}$ or *En1*$^{Cre/+}$; *Otx2*$^{flox/flox}$ + *pCPA*, $p > 0.05$). Saline-treated animals of all genotypes never showed any sign of seizure activity (data not shown).

	Otx2$^{flox/flox}$	*En1*$^{Cre/+}$; *Otx2*$^{flox/flox}$	*En1*$^{Cre/+}$; *Otx2*$^{flox/flox}$ + pCPA	*Otx2*$^{flox/flox}$ + pCPA
number of animals with generalized (stage 4-6) seizures	10/10	1/10 [a]	10/10	10/10
latency to 1st generalized (stage 4-6) seizure (min)	18.9 ± 8.7[b]	52[c]	25 ± 7.9	32.3 ± 12.7

Table 3. Effect of 5-HT depletion on KA seizures in control and *Otx2* conditional mutant mice. [a] The number of animals with generalized seizures significantly differed between *En1*$^{Cre/+}$; *Otx2*$^{flox/flox}$ and the other groups (z-test, $p < 0.001$). [b] Latency to the 1st generalized seizure is calculated from the time of KA administration. Values (min) 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, $p=0.062$).[c] The value reported refers to the only animal that showed a generalized seizure.

Expression of c-fos in Otx2 inactivated mice

We next used c-*fos* mRNA *in situ* hybridization to study the pattern of brain activation at 2 hr after KA injection. 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 (Figure 23). Conversely, *En1*$^{Cre/+}$; *Otx2*$^{flox/flox}$ mice pre-treated with *pCPA* showed the same widespread c-*fos* mRNA labeling, as observed in *Otx2*$^{flox/flox}$ mice. Pre-treatment with *pCPA* did not alter the pattern of KA-induced c-*fos* mRNA expression in *Otx2*$^{flox/flox}$ mice (figure 23). Saline-treated animals of both genotypes did not show any c-*fos* mRNA labeling throughout the brain (data not shown).

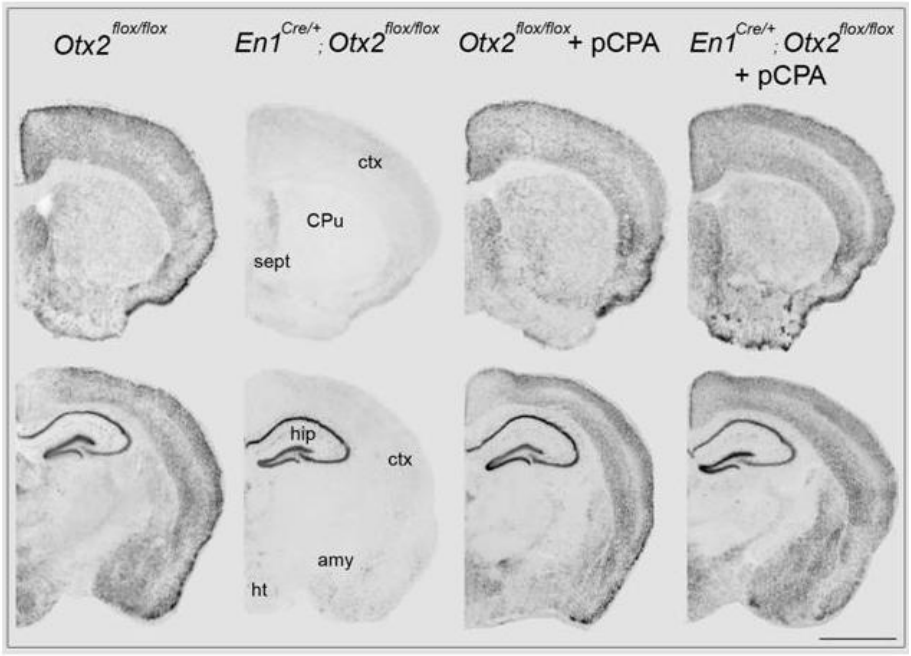


Figure 23. Effect of 5-HT depletion on c-*fos* mRNA expression in the brain of KA-treated control and *Otx2* conditional mutant mice. Panels show c-*fos* mRNA *in situ* hybridizations on coronal sections at the level of the caudate-putamen (top) and dorsal hippocampus (bottom) from representative control and *Otx2* conditional mutant mice (with or without pCPA pre-treatment), 2 hr following 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.

En1$^{Cre/+}$*; tOtx2*^{ov} transgenic mice

KA seizure susceptibility

Adult *En1*$^{Cre/+}$*; tOtx2*^{ov} mice and control *tOtx2*^{ov} mice received a single systemic injection of KA (20 mg/kg) and seizure susceptibility were observed for 3 hrs. *tOtx2*^{ov} (n= 5) mice showed pre-convulsive behavior, with head movements, rigid posture and

facial automatisms in the first half hour (stage 2). Stage 3-4 behaviours (head bobbing followed by isolated limbic motor seizures) appeared after about 60 min following KA administration. This condition lasted for about 1.5 hours. During the 3rd hour, the animals regained some control and began to recover (Figure 24). KA-induced behaviors in *En1*$^{Cre/+}$*; tOtx2*^{ov} mice (n = 8) did not present significant differences compared to control animals. Animals showed pre-convulsive behaviors within thirty minutes after the injection, followed by a worsening of conditions in 60 minutes (stage 4). During the 2nd hours animals showed convulsive behaviors and recovered during the 3rd hour (Figure 24).

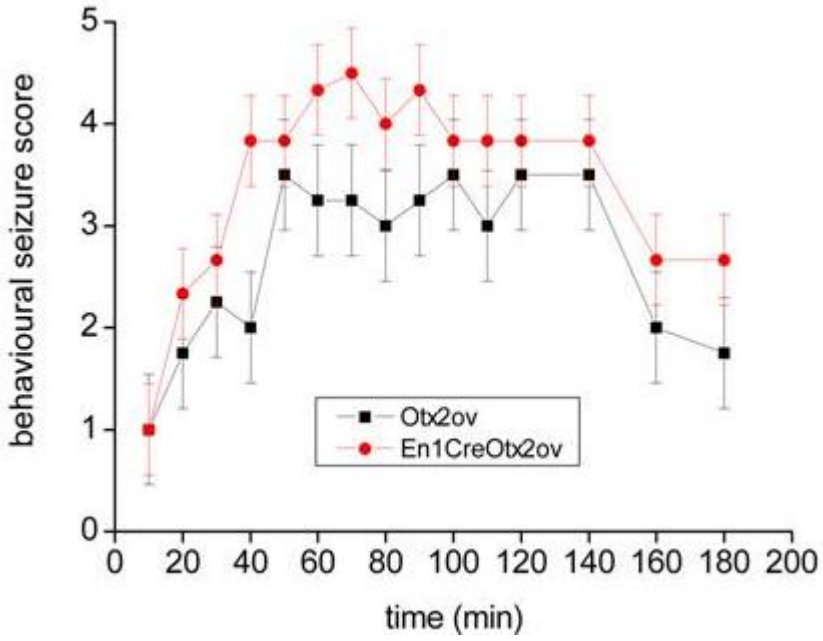


Figure 24. KA seizure susceptibility in Otx2 overexpressing mice. Graph shows the progression of behavioral changes over a 3 hr observation period following KA in *tOtx2*^{ov} (n=5) and *En1*$^{Cre/+}$*;tOtx2*^{ov} mice (n=8). Data are reported as mean seizure scores ± SE.

Expression of *c-fos* mRNA in Otx2 over expressing mice

The expression of c-*fos* mRNA is used as a "marker" of neuronal activity to identify areas involved in epileptic seizures (Willoughby, 1997). The expression of c-*fos* mRNA was analyzed by non-radioactive *in situ* hybridization on sagittal sections from adult brains. In *tOtx2*^{ov} mice treated with KA (stage 4-6 seizures) c-*fos* mRNA expression was throughout the cortex, striatum, thalamus, hippocampus and the entire cerebellum; *tOtx2*^{ov} mice treated with saline only showed a weak signal in the cortex (Figure 25).

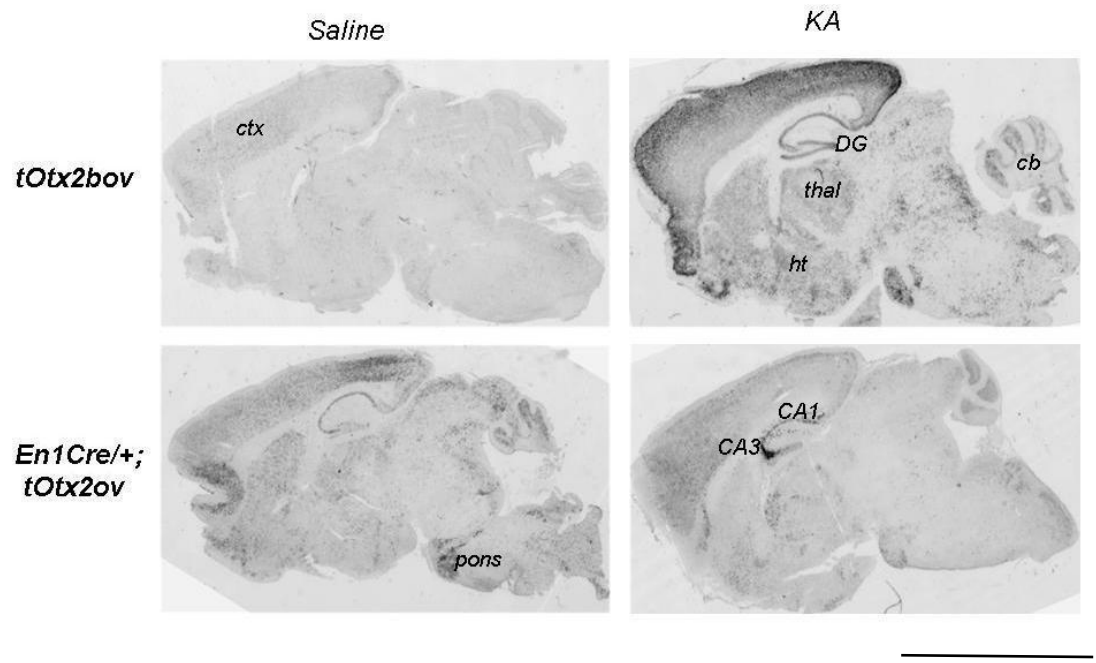


Figure 25. Expression of *c-fos* mRNA expression in the brain of KA-treated control and *Otx2* overexpressing mutant mice. The Panels show c-*fos* mRNA *in situ* hybridizations on coronal sections at the level of the dorsal hippocampus from representative control and *Otx2* over-expressing mice, 2 hr following KA. Genotypes and treatments are as indicated. Abbreviations: cb, cerebellum; ctx, cerebral cortex; DG, dentate gyrus; ht, hypothalamus; thal, thalamus. Scale bar 0.5 cm.

Conversely, saline-treated $En1^{Cre/+}$; $tOtx2^{ov}$ mice showed *c-fos* mRNA expression at the level of temporal cortex, striatum and the thalamus. Interestinglym $En1^{Cre/+}$; $tOtx2^{ov}$ mice treated with KA presented a generally lower c-*fos* mRNA expression throughout the entire brain, with the exception of the CA3 region of the hippocampus (Figure 25).

En-1$^{+/-}$*/En-2*$^{-/-}$ (*En*^{HT}) mutant mice

Seizure susceptibility in *En*^{HT} mice.

Adult *En*^{HT} mice and wild type mice (n=8 per genotype) received a single systemic injection of KA (20 mg/kg) and were observed for 3 hrs. *En*^{HT} mice showed pre-convulsive behaviors (head bobbing and rigid posture) in the initial 20 min after KA administration. Soon after, they reached behavioral seizure score 4-5 (repeated limbic

seizures), and this condition lasted for a period of 90 minutes. Progression of clinical signs in the wild type mice was little less but not significantly different from what observed in En^{HT} mice (Figure 26).

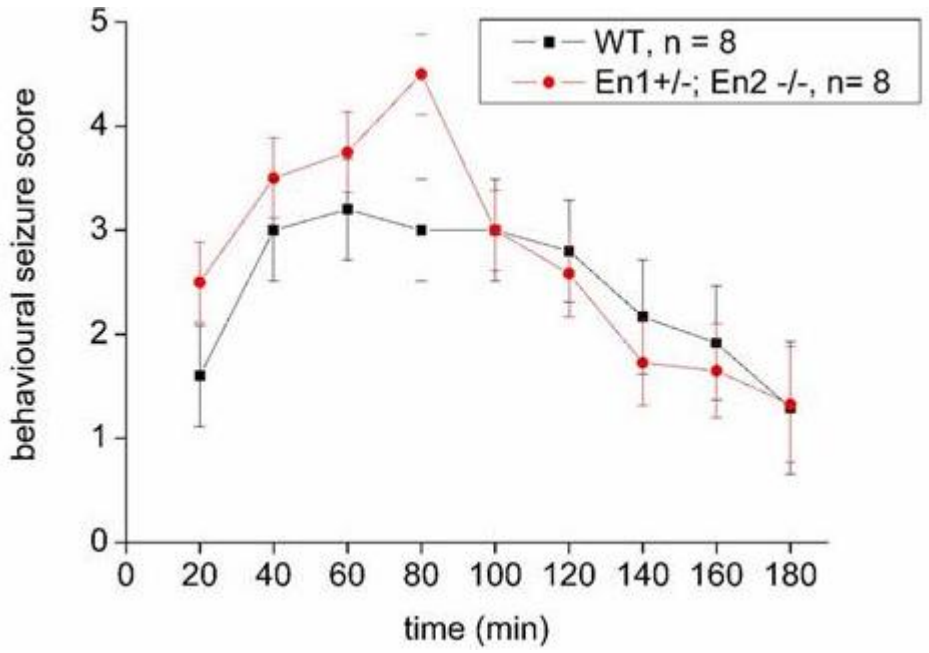


Figure 26. Seizure susceptibilityinduced by KA injection. Graph shows the progression of behavioral changes over a 3 hr observation period following systemic KA administration (20 mg/kg, i.p.) in wild type (n=8) and En^{HT} mice (n=8). Data are reported as mean seizure scores ± SE.

Expression of IEG in En^{HT} mice

We next used c-*fos* mRNA *in situ* hybridization to study the pattern of brain activation at 3 hr after KA in WT and En^{HT} mice. A c-*fos* mRNA labeling was observed in the cerebral cortex, amygdala, thalamus and hippocampus of WT mice, whereas weak c-*fos* mRNA induction was restricted only to the hippocampus (CA3 region) in En^{HT} mice. Saline-treated animals of both genotypes did not show any c-*fos* mRNA labeling throughout the brain (Figure 27).

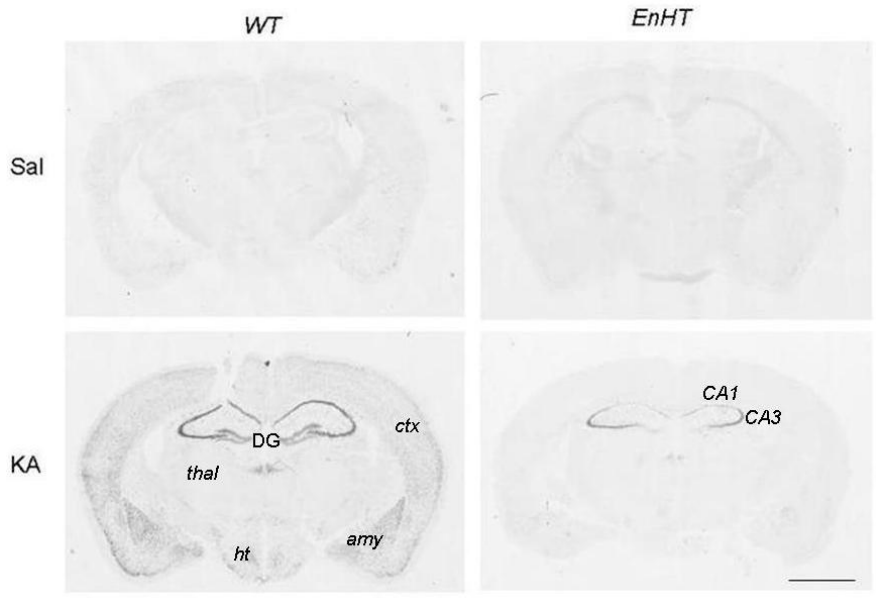


Figure 27. Expression of *c-fos* mRNA expression in the brain of KA-treated control and En^{HT} mutant mice. The images show that c-*fos* mRNA labelling (in black), 3 hr following KA. Scale bar = 2mm. Abbreviations?

En2$^{-/-}$ mutant mice

En2$^{-/-}$ mice display an increased susceptibility to KA seizures.

En2$^{-/-}$ mice were initially used used as controls for En^{HT} mice in KA seizure susceptibility experiments. Unexpectedly, *En2*$^{-/-}$ mice showed an increased response to KA (20 mg/kg) when compared to WT (Figure 28) as well as En^{HT} mice (see Figure 26). In WT, this dose of KA generally resulted in the sole appearance of pre-convulsive behaviors at all time-points analyzed (Figure 26 and 28). Only four out of 8 WT mice displayed brief, isolated episodes of limbic motor seizures (rearing with forelimb clonus, stage 4), and never showed tonic-clonic (stage 6) seizures. The same KA dose in *En2*$^{-/-}$ mice elicited clear signs of focal epilepsy (head bobbing) within the first 20 min, rapidly culminating in stage 4 limbic motor seizures. Latency to the first stage 4 seizure did not differ from that observed in WT mice (Table 4). In sharp contrast with WT, the majority (7 out of 12) of *En2*$^{-/-}$ mice displayed severe tonic-clonic seizures (Table 4). *En2*$^{-/-}$ mice showed generalized stage 4-6 seizures for about two hours (40-160 min; Figure 28). Statistical analysis performed by two-way repeated measures

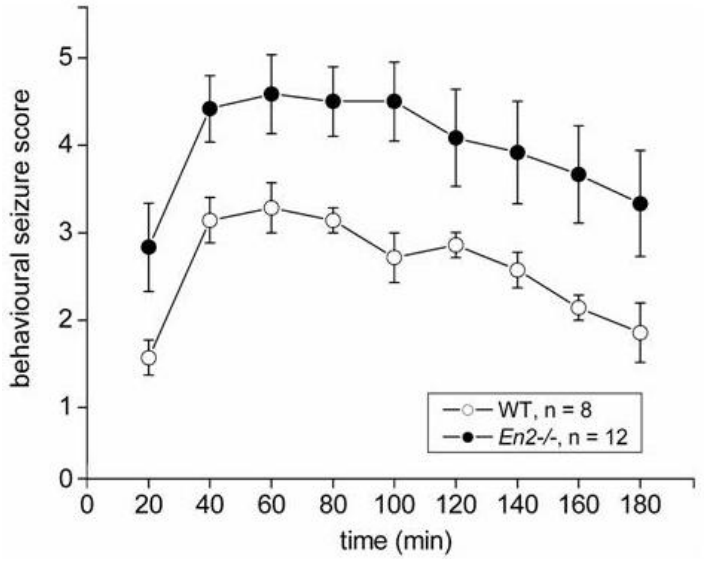


Figure 28. Increased susceptibility to KA-induced seizures in *En2*$^{-/-}$ mice. Progression of behavioral changes after systemic KA administration (20 mg/kg, i.p.) in WT and *En2*$^{-/-}$ over a 3 hr observation period. Data are mean seizure scores ± SE. *, p< 0.05 (post-hoc Tukey test).

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	WT	*En2*$^{-/-}$
animals with stage 4 seizures	4/8	10/12 [a]
latency (min) to 1st stage 4 seizure	35.5 ± 7.7	24.7 ± 3.2 [b]
animals with stage 6 seizures	0/8	7/12 *
latency (min) to 1st stage 6 seizure	n.d.	49.8 ± 13.9

Table 4. Generalized seizures in WT and *En2*$^{-/-}$ mice. Seizure latency (mean ± SE) is calculated from the time of KA administration. [a] not different between the two groups (z-test, $p > 0.05$); [b] not different between WT and *En2*$^{-/-}$ mice (Mann-Withney test, $p > 0.05$); * significantly different between the two groups (z-test, $p < 0.05$); n.d., not determined.

ANOVA revealed a significant effect of genotype ($F_{(1,136)} = 7.522$, $p = 0.014$). Multiple comparison procedure showed that *En2*$^{-/-}$ mice had significantly higher behavioral scores than WT mice at all time points analyzed (Figure 28; Holm-Sidak post-hoc-test, WT vs. *En2*$^{-/-}$ mice, $p < 0.05$). Saline-injected animals of both genotypes showed no sign of epileptic activity during the whole period of observation (not shown).

***En2*$^{-/-}$ mice display increased induction of IEGs after KA seizures.**

c-*fos* and c-*jun* mRNA induction was analyzed by *in situ* hybridization on WT and *En2*$^{-/-}$ brains, 3 hr after KA administration, to map brain areas differentially activated by KA in the two genotypes. In WT mice, c-*fos* mRNA was mainly detected in the hippocampus and in other limbic areas (amygdala, pyriform/entorhinal cortex). No signal was detected in the caudate-putamen and thalamus (Figure 29A). This induction profile was also observed for c-*jun* mRNA, with the exception of the pyriform cortex (that showed no c-*jun* mRNA labelling) (Figure 29A).

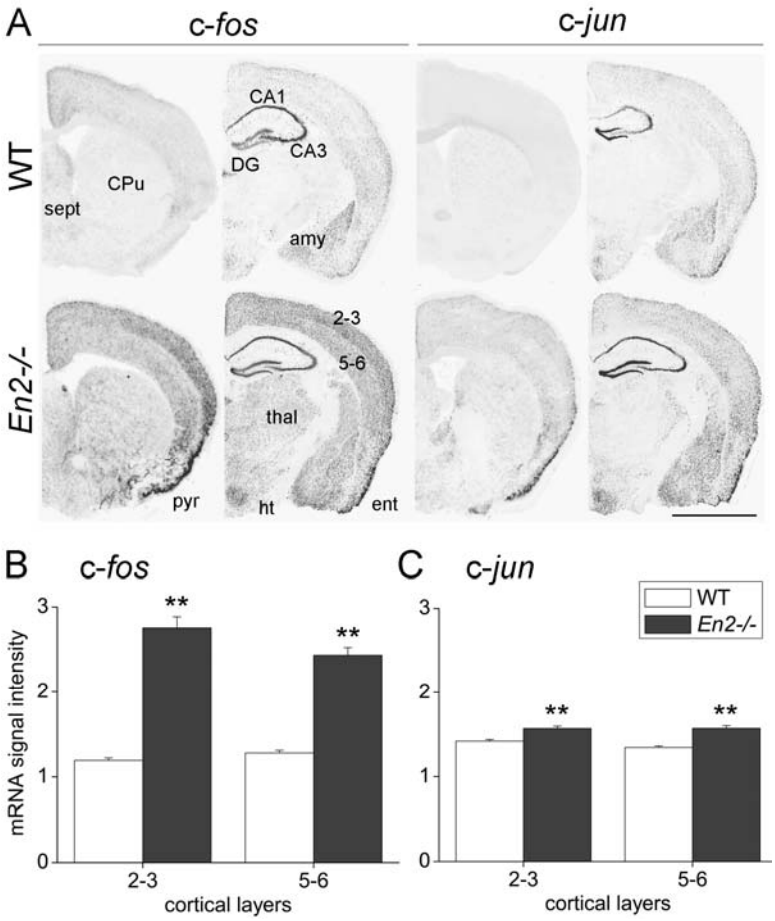

Figure 29. IEGs are differentially induced by KA in WT and *En2*$^{-/-}$ mice. A) c-*fos* and *c-jun* mRNA *in situ* hybridizations, 3h after KA. Representative sections at the level of the caudate-putamen and dorsal hippocampus are shown. Genotypes and relevant brain areas are as indicated. Abbreviations: amy, amygdala; CA1/CA3, pyramidal cell layers of the hippocampus; CPu, caudate-putamen; DG, dentate gyrus; ent, entorhinal cortex; ht, hypothalamus; pyr, pyriform cortex; sept, septum; thal, thalamus. 2-3 and 5-6 indicate layers of the cerebral cortex Scale bar = 2 mm. B,C) Quantification of c-*fos* (B) and c-jun (C) mRNAs in parietal/temporal cortex of KA-treated WT and *En2*$^{-/-}$ mice. Values are mean normalized signal intensities ± SE. **, $p<0.001$ (Student's t-test).

In keeping with seizure generalization observed by behavioral analysis, a widespread and strong induction of both c-*fos* and c-*jun* mRNAs was detected throughout *En2*$^{-/-}$ brains. In particular, c-*fos* and c-*jun* mRNA labelling was evident in caudate-putamen, pyriform cortex, thalamus, amygdala, hippocampus, entorhinal cortex and other cortical areas (Figure 29A). Quantification of *in situ* hybridization experiments performed at the level of the parietal cortex confirmed that c-*fos* and c-*jun* mRNAs were significantly increased in both layers 2-3 and 5-6 in *En2*$^{-/-}$ brains, as compared to WT controls ($p<0.001$, Student's t-test) (Figure 29B-C) Saline-treated mice of both genotypes did not show any c-*fos* mRNA signal, while basal levels of c-*jun* mRNA were detected in the hippocampus in these animals (not shown).

Long-term histopathology in KA-treated *En2*$^{-/-}$ mice.

In order to assess whether increased susceptibility to KA-induced seizures in *En2*$^{-/-}$ mice also resulted in increased susceptibility to long-term damage, the histology of pyramidal cell layers and mossy fiber pathway was evaluated in the hippocampus of WT and *En2*$^{-/-}$ mice, 7 days after KA. Immunostaining for the pan-neuronal marker NeuN on brain sections from WT mice did not reveal any damage in the CA1 pyramidal cell layer (Figure 30), and scattered degenerated cells were only occasionally observed in CA3 pyramidal neurons (Table 5). Conversely, in *En2*$^{-/-}$ mice, cell loss and tissue sclerosis were detected in 4 out of 7 animals in both CA1 and CA3 regions (Figure 30 and Table 5).

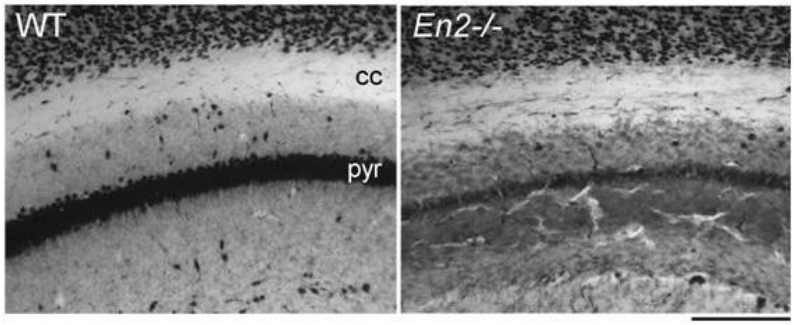

Figure 30. KA-induced neurodegeneration in the CA1 subfield of *En2*$^{-/-}$ mice. NeuN immunostaining of coronal sections from the dorsal hippocampus of WT and *En2* $^{-/-}$ mice, 7 days after KA. Abbreviations: cc, corpus callosum; pyr, pyramidal cell layer. Scale bar: 150 μm.

Histological changes in the mossy fiber pathway were evaluated using neuropeptide Y (NPY) immunohistochemistry. A robust up-regulation of NPY immunoreactivity was found in the mossy fibers of dentate gyrus in all *En2*$^{-/-}$ mice (Figure 31B), whereas no such labelling was detected in WT animals (Figure 31A). Quantification of NPY staining in the mossy fiber pathway from WT and *En2*$^{-/-}$ mice confirmed these findings (Figure 31C). Saline-treated mice of both genotypes did not show any sign of hippocampal histopathology (not shown).

	Degree of cell damage (n. of animals)			
Brain area / genotype	none	little	mild	severe
CA1	5	0	0	0
WT (n = 5)	3	2	1	1
En2$^{-/-}$ (n = 7)				
CA3	3	1	1	0
WT (n = 5)	3	2	0	2
En2$^{-/-}$ (n = 7)				

Table 5. Cell damage in hippocampal CA1/CA3 pyramidal layers of KA-treated WT and *En2*$^{-/-}$ mice. Brain damage was evaluated in NeuN-stained sections, 7 days after KA, according to the scale described in Experimental Methods.

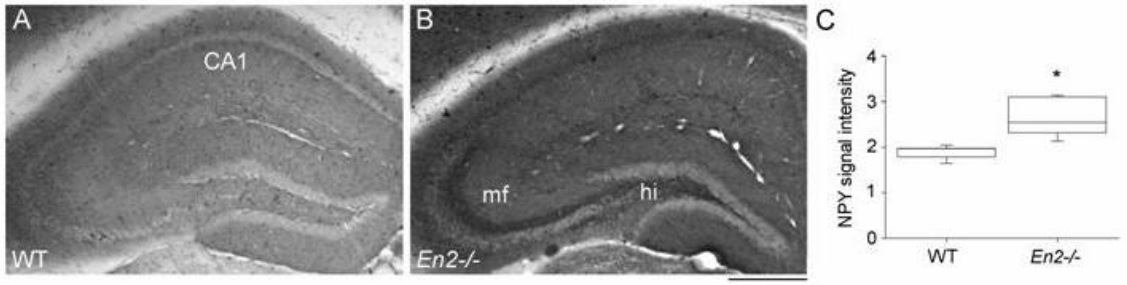

Figure 31. NPY up-regulation in the mossy fiber pathway of KA-treated *En2*$^{-/-}$ mice. A,B) Representative NPY staining in the hippocampus of WT (A) and *En2*$^{-/-}$ (B) mice, 7 days after KA. The almost complete loss of CA1 pyramidal cell layer is also visible in (B). Abbreviations: CA1, pyramidal cell layer; h, hilus; mf, mossy fibers. Scale bar: 500 μm. C) Quantification of NPY staining intensity in the mossy fibers of WT and *En2*$^{-/-}$ mice. Each box chart summarizes the distribution of the NPY signal-to-background ratio (intensity of NPY label divided by the background staining) for all hippocampal

sections in each group. The horizontal lines in the box denote the 25th, 50th, and 75th percentile values. The error bars denote the 5th and 95th percentile values. **, $p < 0.001$ (Mann-Whitney rank sum test).

En2 mRNA is expressed in the adult mouse hippocampus and cerebral cortex.

Neuroanatomical and behavioural studies performed on adult *En2*$^{-/-}$ mice suggest that *En2* might be expressed also in anterior brain structures during adulthood (Cheh *et al.* 2006; Kuemerle *et al.* 2007). We therefore investigated *En2* mRNA expression in different brain areas of the adult mouse brain. To this purpose, we performed quantitative real-time RT-PCR experiments using the mitochondrial ribosomal L41 protein mRNA as a standard for quantification.

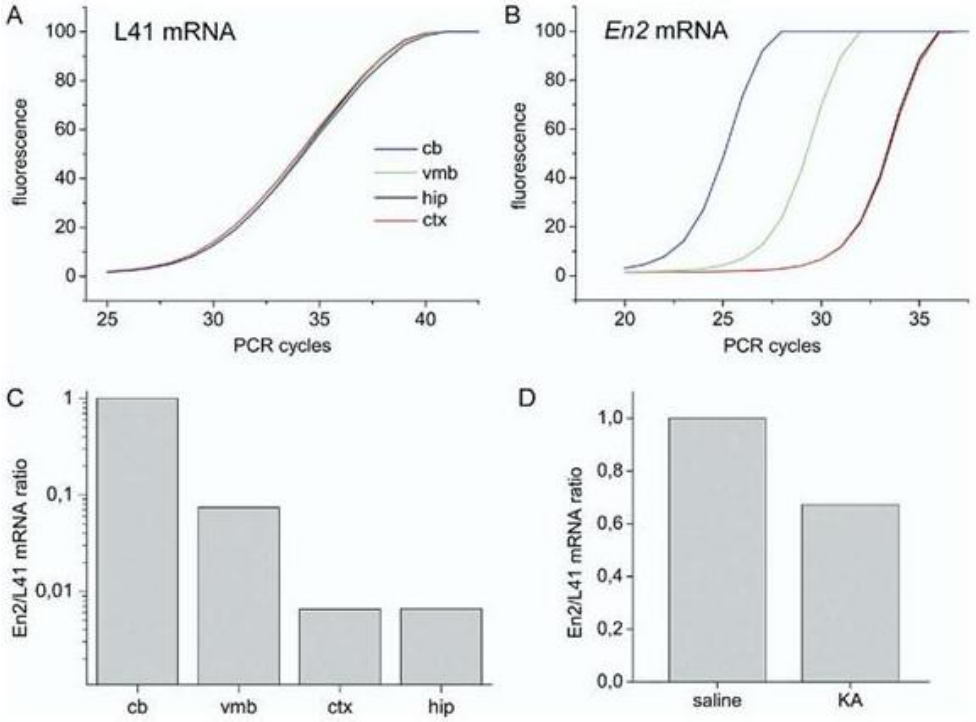

Figure 32- *En2* mRNA is expressed in adult mouse hip and ctx and is regulated by seizure activity. (A, B) Real-time RT-PCR amplification profiles of mitochondrial ribosomal protein L41 (A) and *En2* (B) mRNAs from the cb (blue line), vmb (green line), hip (black line) and ctx (red line) of WT adult mice. The graphs report the appearance of fluorescence in PCR amplicons as a function of the number of PCR cycles. (C, D) Graphs report the quantification of real-time RT-PCR experiments. In (C), values are expressed as En2 mRNA/L41 mRNA comparative quantitation ratios (average values of three technical replicates) in different adult brain areas, normalized to cb. In (D), values are expressed as *En2* mRNA/L41 mRNA comparative quantitation ratios (average values of three technical replicates), from the hip of KA-treated adult mice (3 h post KA, 20 mg/kg i.p.), normalized to saline-treated controls. Abbreviations: cb, cerebellum; ctx, cerebral cortex; hip, hippocampus; vmb, ventral midbrain.

As expected, L41 amplification gave comparable amplification curves from all brain areas analyzed (cere- bellum, ventral midbrain, hippocampus, cerebral cortex; Figure 32A). *En2* transcripts were detected at low but significant levels in the hippocampus and cerebral cortex; En2 amplification curves from these two areas were indistinguishable (Figure 32B). According to previous studies (Joyner *et al.* 1991; Millen *et al.* 1994; Simon *et al.* 2001), *En2* mRNA was detected at higher levels in the ventral mid-brain and cerebellum (Figure 32B). Comparative quantification of real-time RT-PCR experiments showed that in hippocampus and cerebral cortex, En2 mRNA was present about 100 times less than in cerebellum (Figure 32C). In the adult hippocampus, *En2* mRNA levels were regulated by pathological hyperactivity. In animals that experienced generalized seizures following systemic administration of the glutamate agonist KA (20 mg/kg i.p.), *En2* mRNA levels were decreased by 30%, as compared to saline-treated controls (Figure 32D).

$En2^{-/-}$ mice have a reduced expression of GABAergic markers in the hippocampus, cerebral cortex and hilus.

Since *En2* is expressed in the adult hippocampus (Figure 32B, C), we sought to investigate the presence of subtle neuro-anatomical defects in this structure of $En2^{-/-}$ mice. Immunohistochemistry experiments with anti-parvalbumin and anti-somatostatin antibodies were performed to detect selected GABAergic interneuron populations (Matyas *et al.* 2004; Jinno and Kosaka, 2006) in the hippocampus of WT and $En2^{-/-}$ mice. In WT mice, parvalbumin revealed the typical staining around the cell bodies of pyramidal neurons of CA1 (not shown) and CA3 (Figure 33) hippocampal subfields (see also Matyas *et al.* 2004), whereas somatostatin predominantly labeled stratum lacunosum moleculare and hilar interneurons (Fig. 33; see also Matyas *et al.* 2004). In $En2^{-/-}$ mice, staining for both parvalbumin and somatostatin was markedly reduced in CA3 pyramidal layer and stratum lacunosum moleculare, respectively (Figure 33).

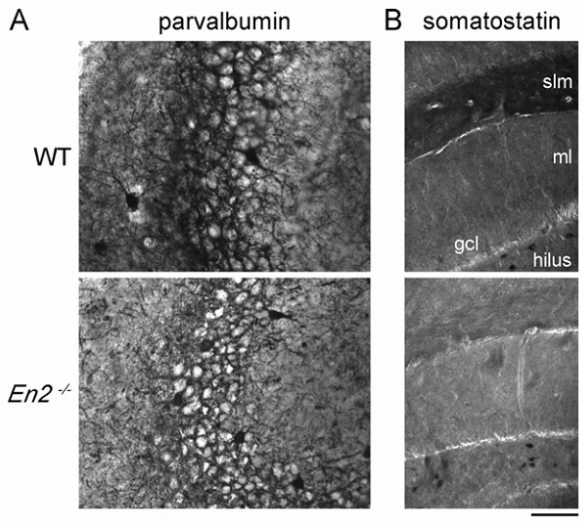

Figure 33. Downregulation of GABAergic markers in the hippocampus of *En2*$^{-/-}$ mice. (A) Representative parvalbumin staining in the CA3 pyramidal cell layer of WT and *En2*$^{-/-}$ mice. (B) Representative somatostatin staining in the slm and hilus of WT and *En2*$^{-/-}$ mice. Abbreviations: gcl, granule cell layer; ml, molecular layer; slm, stratum lacunosum moleculare. Scale bar 50 μm.

We further investigated the neuroanatomy of GABAergic interneurons in the hippocampus and cerebral cortex of adult *En2*$^{-/-}$ mice by performing immunohistochemistry for parvalbumin (PV), NPY and somatostatin (SOM). Adult (6 months old) *En2*$^{-/-}$ mice, when compared to their age-matched WT controls, present a statistically significant reduction in the number of PV-positive interneurons in layers 2-3 of the parietal/temporal cortex and in the hilus of the dentate gyrus but no significance difference was observed in layers 5-6 of the parietal/temporal cortex (Figure 34). A statistically significant reduction in the number of NPY-positive interneurons was also detected in the parietal/temporal cortex (layers 2-3 and 5-6) as well as the hilus of *En2*$^{-/-}$ mice, as compared to WT (Figure 34). Similarly, the significant reduction in the number of SOM-positive interneurons was observed in *En2*$^{-/-}$ mice in layers 2-3 of the parietal/temporal cortex when compared with the WT animals, but no significance difference was observed in layers 5-6 of the parietal/temporal cortex and hilus of the dentate gyrus (Figure 34).

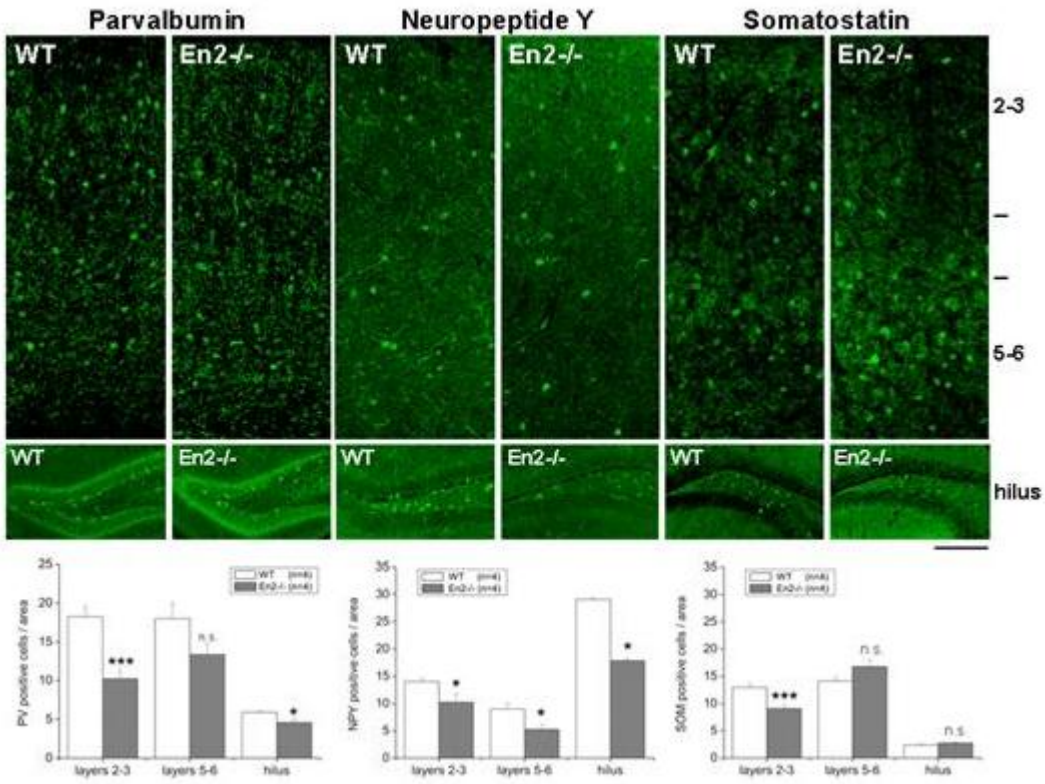

Figure 34. Reduction of parvalbumin, NPY and somatostatin positive neurons in the cerebral cortex and hilus of *En2*$^{-/-}$ mice. Top: representative PV, NPY, SOM immunostainings from the parietal/temporal cortex of WT and *En2*$^{-/-}$ mice. Cortical layers are indicated. Middle: representative PV, NPY, SOM positive neurons in the hilus of WT and *En2*$^{-/-}$ mice. Bottom: quantification of PV, NPY, SOM immunohistochemistry data. Data are expressed as the mean number of positive cells per area +/- s.e.m (area was 0.06 mm^2 for cortical layers and 0.1 mm^2 for the hilus). *, $p< 0.05$; ***, $p< 0.001$ (t-test, *En2*$^{-/-}$ vs. WT). n.s., not significant difference. Scale bar: 100 μm.

DISCUSSION

Summary of results

Our studies, performed on classical and conditional knockout mouse lines, demonstrate that altered embryonic development of dopaminergic and serotonergic neurons results in altered seizure susceptibility in the adult life.

We investigated seizure susceptibility in the following mutant mice.

1) Mice with conditional inactivation of the Otx2 gene in DA precursor cells. In these mice, Otx2 was conditionally inactivated by a Cre recombinase expressed under the transcriptional control of the En1 gene (*En1*$^{Cre/+}$*; Otx2*$^{flox/flox}$). Severe abnormalities were detected in the ventral midbrain, namely extensive reduction and disorganisation of DA neurons. This resulted in a neurotransmitter fate switch from DA to 5-HT so that these mice had a 70% reduction in the number of DA neurons and an increased number of 5-HT neurons in the ventral midbrain, that persisted until adult age (Puelles *et al.* 2004; Borgkvist et al., 2006). In particular, adult *En1*$^{Cre/+}$*;Otx2*$^{flox/flox}$ mice showed a massive increase of 5-HT in the pons, ventral midbrain, hippocampus (CA3 subfield) and cerebral cortex, that was paralleled by reduced levels of 5-HT transporter (Sert) in the same areas. Due to this increased 5-HT hyper-innervation, *En1*$^{Cre/+}$*; Otx2*$^{flox/flox}$ mice were resistant to generalized seizures induced by the glutamate agonist kainic-acid (KA). Brain 5-HT depletion by *pCPA* in conditional mutant mice reduced 5-HT content to control levels in these brain areas, fully re-establishing KA-seizure susceptibility, meaning that increased brain 5-HT levels were responsible for seizure resistance in Otx2 conditional mutants.

2) Mice with conditional over-expression of the Otx2 gene in DA precursor cells. In these mice, Otx2 was conditionally overexpressed by a Cre recombinase under the transcriptional control of the En1 gene (*En1*$^{Cre/+}$*; tOtx2*^{ov}). Otx2 overexpression resulted in a 35% increase of DA progenitors neurons in the VTA of the anterior,

and more prominently in posterior mesencephalon (Omodei *et al.* 2008). It is important to point out that, apart from the increased number of DA progenitors, these conditional over-expressing mice did not show any alteration in 5-HT neurons. The increase in DAergic cell number persisted until adult age. $En1^{Cre/+}$; $tOtx2^{ov}$ mice did not show significantly altered KA induced seizure susceptibility when compared to control animals.

3) $En1^{+/-}$; $En2^{-/-}$ (En^{HT}) mice, which display a postnatal and progressive loss of DA neurons of the substantia nigra. The phenotype of these mutant mice resembles key pathological features of Parkinson's disease. It is important to point out that the postnatal DA cell loss in En^{HT} mice is not accompanied by altered number of 5-HT cells. En^{HT} mice did not show significantly altered KA induced seizure susceptibility when compared to control animals.

4) $En2^{-/-}$ mice, which show no alteration in the number of DA and 5-HT neurons at all ages, were initially used as a control strain for experiments performed on En^{HT} mice. When we evaluated KA seizure susceptibility in $En2^{-/-}$ mice, we surprisingly and unexpectedly found that $En2^{-/-}$ mice have an increased susceptibility to KA-induced seizures. $En2^{-/-}$ mice also showed long-term histopathology with marked degeneration in CA1 pyramidal neuron and up-regulation of mossy fiber pathway. The occurrence of generalized seizures in $En2^{-/-}$ mice was likely due to defects in GABAergic innervation onto principal hippocampal neurons and reduced number of inhibitory neurons (such as SOM, PV and NPY-positive neurons) in the hilus and cortical layers 2-3 and 5-6.

DA, 5-HT and seizure susceptibility

Systemic KA administration has been widely used to study the susceptibility to acute seizures and seizure-induced long-term histopathology in inbred and mutant mouse strains. Our studies, carried out in mutant mouse lines with alteration in Otx2 or Engrailed1/2 genes, show that altered specification of DA and 5-HT cell fate during embryonic development results in altered seizure susceptibility in the adult age.

Classical pharmacological studies indicate that both DA and 5-HT may have an anti-epileptic action.

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). Indeed the neuroprotective role of DA D2 receptors against glutamate-induced excitotoxicity has been well described (reviewed in Bozzi and Borrelli, 2006). DA through D2 receptor exerts an inhibitory control on the response to seizure promoting stimuli such as KA (Bozzi et al. 2000). Mesolimbic DAergic pathways have been proposed to exert this inhibitory control (LaGrutta and Sabatino, 1990; Starr, 1996). Similarly, there has been increasing evidence that serotonergic neurotransmission modulates a wide variety of experimentally induced seizures. Generally, agents that elevate extracellular 5-HT levels, such as 5-hydroxytryptophan and serotonin reuptake blockers, inhibit both focal and generalized seizures. Conversely, depletion of brain 5-HT lowers the threshold to audiogenically, chemically and electrically evoked convulsions (Badgy *et al.* 2007). Furthermore, it has been shown that several anti-epileptic drugs such as valproic acid, lamotrigine, carbamazepine, phenytoin, zonisamide elevate and/or stimulate basal 5-HT levels and/or release, as part of their anticonvulsant action (Okada *et al.* 1992; Dailey *et al.* 1996; Ahmad *et al.* 2005). Moreover, 5-HT receptors are expressed in almost all networks involved in epilepsies. These studies indicate that both DA and 5-HT are clearly involved in the control of epileptic seizures. According to this view, it was expected that reduction of DA cells in both $En1^{Cre/+}; Otx2^{flox/flox}$ and En^{HT} mice would contribute to increase seizure susceptibility in these animals, while increase in DA cells in $En1^{Cre/+}; tOtx2^{ov}$ mice would contribute to lower seizure susceptibility severity. On the contrary, $En1^{Cre/+}; Otx2^{flox/flox}$ mice were markedly resistant to KA seizures due to 5-HT hyper-innervation, whereas $En1^{Cre/+}; tOtx2^{ov}$ mice and En^{HT} mice (in which 5-HT levels were unchanged) showed a normal susceptibility to KA induced seizures (Table 6). This is in line with earlier observation that 5-HT levels are inversely proportional to seizure susceptibility. More importantly, altered level of DA in $En1^{Cre/+}; Otx2^{flox/flox}$, $En1^{Cre/+}; tOtx2^{ov}$ and En^{HT} mice had less impact in altering seizure susceptibility.

Indeed *En1*$^{Cre/+}$*; Otx2*$^{flox/flox}$ mice (which have reduced level of DA) did not show increased seizure susceptibility, while *En1*$^{Cre/+}$*; tOtx2*^{ov} and *En*^{HT} mice (which have higher and lower level of DA, respectively, with no alterations in 5-HT), showed unaltered seizure threshold. Thus, the altered embryonic development of 5-HT neurons seems to have a more prominent effect onto seizure control than the altered development of DA neurons (Table 6: Tripathi *et al.* 2010).

Mouse strain	DA and 5-HT alterations	KA seizure susceptibility
En1$^{Cre/+}$*; Otx2*$^{flox/flox}$	Less DA, more 5-HT	Resistant
En1$^{Cre/+}$*; tOtx2*^{ov}	More DA, no difference 5-HT	Not altered
En1$^{+/-}$*; En2*$^{-/-}$ (*En*^{HT})	Less DA, no difference 5-HT	Not altered
En2$^{-/-}$	No difference DA, 5-HT	Increased

Table 6. The effect of 5-HT hyper-innervation onto seizure control is more prominent than that of DA reduction in these animal models.

The brain areas which are crucially involved in the serotonergic control of seizures are the ventral midbrain and limbic system. 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 pre-treatment 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. Indeed, 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.

Impact of genetic background on seizure susceptibility

It is widely known that the genetic background impacts seizure susceptibility in the mouse. Specifically, several studies demonstrate that different inbred mouse strains have a different response to KA-induced seizures. For example, DBA/2 mice are extremely prone to KA-induced seizures, whereas C57Bl/6 have a relatively lower susceptibility when compared to DBA/2 (Ferraro *et al.* 1995; Schauwecker and Steward, 1997). In our study, we used $Otx2^{flox/flox}$, $En1^{cre/+}$; $Otx2^{flox/flox}$, $tOtx2^{ov}$ and $En1^{Cre/+}$; $tOtx2^{ov}$ mice of a mixed DBA/2 x C57BL/6 genetic background, while En^{HT}, $En2^{-/-}$ and WT mice were of a mixed 129/Sv x C57BL/6 genetic background.

$Otx2^{flox/flox}$ control mice displayed high susceptibility to seizures induced by 20 mg/kg KA, similarly to what has been observed in DBA/2 mice (Ferraro *et al.* 1995; McLin and Steward, 2006) and mice with a mixed DBA/2 x C57BL/6 background (Dell'Agnello *et al*. 2007). Conversely, $En1^{cre/+}$; $Otx2^{flox/flox}$ mice showed a marked resistance to KA induced seizures, indicating a protective effect of 5-HT in the mixed DBA/2 x C57BL/6 background. This also strengthens the idea that the effect of 5-HT hyper-innervation onto seizure control is more prominent than that of DA reduction.

Since $En1^{Cre/+}$; $tOtx2^{ov}$ mice have a mixed DBA/2 x C57BL/6 genetic background, one would have expected higher susceptibility to KA-induced seizures in these mice. However, $En1^{Cre/+}$; $tOtx2^{ov}$ mice showed moderate susceptibility to seizures induced by KA. This can be explained with the fact that conditional overexpessing mice have an increased number of DA in VTA but no alteration in 5-HT neurons. Indeed, in the experiments with Otx2 conditional mutants we have shown that altered DA levels have no impact onto seizure susceptibility.

In the experiments with $En1^{+/-}$; $En2^{-/-}$ (En^{HT}) and $En2^{-/-}$ mice we used animals of amixed 129/Sv x C57BL/6 genetic background. As expected, WT mice of this background displayed a very mild response to 20 mg/kg KA, never showing continuous generalized epileptic activity (stage 5 seizures) or tonic-clonic seizures (stage 6) after KA. The response of En^{HT} mice to the same KA dose did not differ from that of WT controls; this is in keeping with our idea that altered DA cell number has a minimal influence onto seizure control. Unexpectedly, $En2^{-/-}$ mice of this background showed higher seizure susceptibility; the importance of these results is discussed below.

IEGs study in the different mouse strains

The striking activation of immediate early genes (IEGs) like *c-fos* and c-jun expression in neurons following seizures (Morgan *et al.* 1987) led to consider the induction of these genes as a marker of neuronal activity in the mammalian nervous system (Sagar *et al.* 1988). A precise correlation exists between the appearance of generalized seizures following KA and the pattern of c-*fos* and *c-jun* mRNA induction. Pre-convulsive behaviors (stages 1-3 of the 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 al.* 2000). Seizure resistance in $En1^{cre/+}$; $Otx2^{flox/flox}$ mice was indeed confirmed by gene expression studies. $En1^{cre/+}$; $Otx2^{flox/flox}$ mice never experienced generalized seizures after KA, showing *c-fos* mRNA induction restricted to the hippocampus. Conversely, $En1^{cre/+}$; $Otx2^{flox/flox}$ mice pretreated with *pCPA*, as well as $Otx2^{flox/flox}$ mice, showed KA-induced generalized seizures and widespread induction of c-*fos* mRNA from hippocampus to different brain region including caudate putamen, septum, cerebral cortex, amygdala and hypothalamus (Figure 23; Tripathi *et al.* 2008). Pre-treatment with *pCPA* did not alter the pattern of KA-induced c-*fos* mRNA expression in $Otx2^{flox/flox}$ mice.

Mild susceptibility to KA seizures in $tOtx2^{ov}$ control mice correlated with moderate to high *c-fos* mRNA expression in temporal and parietal cortex, thalamus, hippocampus and cerebellum. Conversely, conditional overexpression of Otx2 in $En1^{Cre/+}$; $tOtx2^{ov}$ mice resulted in a reduced expression of *c-fos* mRNA in these brain regions following KA treatment. This suggests that the incresased VTA cell number (and, likely, increased DA projections to the limbic system) has an inhibitory effect onto KA-dependent c-*fos* mRNA expression in $En1^{Cre/+}$; $tOtx2^{ov}$ mice. These data definitely need further investigation.

En^{HT} mice have a moderate KA seizure susceptibility and show a weak c-*fos* mRNA induction restricted only to the hippocampus (mainly in the CA3 region). It is interesting to note that apart from the reduced number of DA neuron, En^{HT} mice have lower levels of the dopamine transporter (DAT) (Paola Sgadò, personal communication) meaning that synaptic DA level might not be significantly less or

different than control animals. This might explain the unaltered seizure susceptibility observed in these mice (Figure 26). Indeed DAT may play an important role in modulating gene expression and contributing to neuroadaptive processes within dopamine neurons. The DAT substrates dopamine and amphetamine robustly induced *c-fos* expression in hDAT cells but not in wild-type HEK-293 cells, demonstrating that that dopamine can induce *c-fos* expression in a DAT-dependent manner (Yatin *et al*. 2002). In accordance with this, lower level of DAT might contribute also in reduced *c-fos* mRNA expression in En^{HT} mice.

$En2^{-/-}$ mice showed long-lasting generalized seizures and widespread induction of IEGs like *c-fos* and *c-jun* mRNA in caudate-putamen, pyriform cortex, thalamus, amygdala, hippocampus, entorhinal cortex and layer 2-3 and layer 5-6 of cortical areas. As expected, WT C57BL/6 mice experienced no or very brief generalized seizures after KA, showing IEGs mRNA induction restricted to the hippocampus and other limbic areas (Figure 29; Tripathi *et al.* 2009).

Long-term damage

In order to assess whether increased susceptibility to KA-induced seizures in $En2^{-/-}$ mice also resulted in increased susceptibility to long-term damage, the histology of pyramidal cell layers was evaluated in the hippocampus of WT and $En2^{-/-}$ mice, 7 days after KA. It is well known that CA1 and CA3 pyramidal neurons are the most vulnerable to the excitatory and neurotoxic effects of KA (Ben-Ari, 1985) because of the high density of KA binding sites in this terminal field of the hippocampal mossy fibre system (Berger and Ben-Ari, 1983). Indeed, CA1 pyramidal cells of KA-treated rats show increased *N*-methyl-D-aspartate (NMDA) excitatory postsynaptic responses (Turner and Wheal 1991; Williams *et al.* 1993) while GABA mediated inhibitory synaptic responses are diminished in CA1 pyramidal cells of KA-treated rats (Ashwood *et al.* 1986; Franck and Schwartzkroin 1985; Franck *et al.* 1988). Generally, mouse strains derived from the C57BL/6 strain show minimal cell death in pyramidal hippocampal neuron and are resistant to mossy fibre sprouting (McLin and Steward, 2006). In keeping with these findings, our WT mice never showed cell damage in the CA1 pyramidal layer and only occasionally presented little or mild damage in the CA3.

A marked degeneration was instead observed in all *En2*$^{-/-}$ mice analyzed in the CA1 and, to a lesser extent, in the CA3. The variability in the degree of brain damage following systemic KA administration that we observed in WT and En2-/- mice is in agreement with previous studies (Schauwecker and Steward, 1997; Bozzi *et al.* 2000; McLin and Steward, 2006). NPY is particularly abundant in the dentate gyrus. In this region, it is normally expressed mainly by a subset of hilar GABA neurons. It is believed that mossy fibre sprouting after KA-induced seizures occurs in response to the denervation of the inner molecular layer as a result of the death of neurons in the hilus of the dentate gyrus (Cantallops & Routtenberg, 2000). For this purpose, we studied NPY up-regulation in the mossy fiber pathway (dentate gyrus to CA3). Importantly, *En2*$^{-/-}$ but not WT mice treated with KA displayed NPY up-regulation in the mossy fiber pathway. This pattern of NPY up-regulation is generally considered a reliable marker of long-term, post-seizure synaptic rearrangements, and is thought to be indicative of an acquired hyper-excitability of the hippocampus following seizures (Morimoto *et al.* 2004; Nadler *et al.* 2007; Dudek and Sutula, 2007; Sperk *et al.* 2007; Sutula and Dudek, 2007). Moreover, as observed for pyramidal cell loss, rearrangements in mossy fibers do not occur following KA in mouse strains derived from C57BL/6 (Schauwecker *et al.* 2000). Our data suggest that inactivation of the *En2* gene also results in long-term anatomical modifications and, likely, hyper-excitability of hippocampal circuitry in response to KA seizures.

Seizure susceptibility in *En2*$^{-/-}$ mice: the role of GABAergic inhibitory system

En2$^{-/-}$ mice showed increased seizure severity in response to KA. Importantly, spontaneous seizures never occurred in naive *En2*$^{-/-}$ mice (data not shown), indicating that increased susceptibility of *En2*$^{-/-}$ mice is evident only in response to a potent seizure-promoting stimulus, such as KA. We investigated whether this increased response to KA might be due to altered anatomy of inhibitory GABAergic neurons in the hippocampus and cerebral cortex.

Changes in GABAergic inhibition in the hippocampus have been examined in many experimental models of epilepsy. In general, inhibitory cell loss in epileptogenic zones correlates with the induction of seizures, and results in the decrease in synaptic

inhibition in pyramidal neurons (Bekenstein and Lothman 1993; Sloviter 1987). Moreover, a reduced number of GABAergic neurons in epileptogenic areas is a likely cause of hyper-excitability. For this reason, we studied the anatomy of different subpopulations of GABAergic neurons (SOM-, PV- and NPY-positive) in the cerebral cortex and hippocampus of *En2*$^{-/-}$ mice. We observed a reduced staining of all these inhibitory markers in the cerebral cortex (layers 2-3) and hippocampus of *En2*$^{-/-}$ mice, that might explain the hyper-excitability observed in these mutants.

It remains to be determined how the *En2* mutation can impact hippocampal excitability, a point that was not addressed by our study. Indeed, the *En2* gene is commonly known for its crucial role in pattern formation of the midbrain and hindbrain regions. Perhaps due to the more posterior location of engrailed expression, none of the alteration in telencephalic structures has been analyzed in either engrailed mutant. However, novel evidence suggests that *En2* might be implicated also in the development of more anterior telencephalic structures. Kuemerle and coworkers recently reported distinct anterior shift in the position of the amygdala in the cerebral cortex of *En2*$^{-/-}$ mice. Specifically, it was found that in *En2* mice the lateral, basolateral, central and medial nuclei of the amygdala are located in approximately 500 um to a more anterior position in the cortex when compared to controls (Kuemerle et al., 2007). This parallel anterior shift of all four of the nuclei indicates that a noteworthy amygdaloid defect is present in the *En2* mice. Interestingly, Miyazaki *et al.* have reported a more caudal (posterior) shift in the location of 5-HT immunostained cells in the dorsal raphe nucleus of postnatal day 50 rats that were exposed to thalidomide or valoproic acid during early embryogenesis. The authors surmise that the locational shift of the 5-HT cells is most likely the result of aberrant neuronal migration early in development (Miyazaki *et al.* 2005). It might be possible that something similar could be happening in the *En2* mutant. With these studies, it seems apparent that the organization of the CNS places a high premium on developing a proper balance among its many components (Kuemerle *et al.* 2007). These anatomical alterations might impact circuitry and excitability of the amygdala and other limbic structures in *En2*$^{-/-}$ mice. En2 secretion from posterior structures to anterior target areas could explain these effects. Indeed, En-2 may also have a role in cell–cell communication, as suggested by

the presence of other domains involved in nuclear export, secretion and internalization (Joliot *et al.* 1998; Brunet *et al.* 2005; Sonnier *et al*. 2007). Further studies might be aimed at determining whether *En2* released from ventral midbrain projections can influence excitability of more anterior limbic structures such as hippocampus. Our data show that in the adult mouse brain, *En2* is also expressed in the hippocampus and cerebral cortex, two regions crucially involved in seizure generation and spread. These results confirm and expand non-quantitative RT- PCR expression data previously published online in the Mouse Genome Informatics (MGI) database (http://www.informatics.jax.org).

Engrailed-2 and Autism Spectrum Disorders (ASD)

The autism spectrum disorder (ASD) is among the most devastating disorders of childhood, afflicting up to 13 out of every 10,000 individuals (Fombonne *et al.* 2005). ASD comprises several different disorders as defined by deficits in social behaviors and interactions. These deficits prevent the development of normal interpersonal relationships features that typify the broad range of autistic behavior include language impairments (including deficits in verbal and non-verbal communication), restricted patterns of interests and activities, abnormal responses to sensory stimuli, poor eye contact, an insistence on sameness, an unusual capacity for rote memorization, and often repetitive actions (Kemper *et al.* 1998). Although the neuroanatomical basis of autism is still somewhat unclear, certain brain regions appear to be regularly altered in individuals with ASD. These include areas in the neocortex, cerebellum, amygdala, hippocampus and brain stem (Bauman 1991; Bauman *et al.* 1985; Courchesne 1995). It is believed that there are a half dozen or more genes remaining to be discovered, three genes that are emerging as plausible players in the etiology of ASD are reelin (RELN), the serotonin transporter gene (5-HTT), and *En2* (Bartlett *et al.* 2005).

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) and subtle but reproducible disruption in the anterior/posterior pattern of cerebellar foliation and transgene expression, particularly in posterior vermis (Joyner *et al.* 1991; Millen *et al.* 1994). 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), as well as "autistic-like" behaviours, such as decreased attitude to play, reduced aggressiveness and special learning deficits. The cerebellum of *En2*$^{-/-}$ mice is about one-third smaller than its wild type counterpart and harbors subtle abnormalities in its folial pattern. Cell counts reveal that all the major cell types of the olivocerebellar circuit (Purkinje, granule, inferior olive and deep nuclear) are reduced by 30–40% in the *En2*$^{-/-}$ mice (Kuemerle *et al.* 1997). Although the structural and cellular changes in the *En2*$^{-/-}$ cerebellum are not completely congruent with those reported in the autistic brain, they are still quite analogous. At a functional level, Pierce and Courchesne (2001) provide insight as to how cerebellar abnormalities may be linked with autistic behavior.

More recently, altered anatomical structure of the amygdala has also been reported in *En2*$^{-/-}$ mice. In these mice, there is an anterior shift of lateral, basolateral, central and medial amygdalar nuclei reside in the cortex (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, entorhinal cortex and other limbic areas have shown small cell size and increased cell packing density at all ages (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 to ASD, and two single-nucleotide polymorphisms (SNPs) rs1861972 and rs1861973, in the EN2 gene have been associated to ASD (Gharani *et al.* 2004; Benayed *et al.* 2005; Brune *et al.* 2008, Benayed 2009).

A focal brain pathology that affects frontal or mesiotemporal structures (limbic system) can be at the origin of an autistic phenotype as well as the trigger of an epilepsy that

aggravates the autistic symptoms. Epilepsy and epileptic process occurring in early development interferes with the developing function of specific brain networks involved in communication and social behavior (Deonna and Roulet, 2006). Our results showed that *En2*$^{-/-}$ mice have an increased susceptibility to seizures evoked by KA (Tripathi *et al.* 2009). This is in agreement with the high prevalence of epilepsy described in ASD patients (Canitano, 2007). Moreover, *En2*$^{-/-}$ mice display a series of neuroanatomical alterations (i.e., reduced expression of GABAergic markers in the hippocampus) that might underlie increased excitability in these mutants (Figure 33, 34 and Tripathi *et al*. 2009). These data, along with recent findings from other authors (Kuemerle *et al.* 2007), suggests that *En2* is implicated also in the development of telencephalic structures. Kuemerle and coworkers reported an anterior shift of the amygdala in *En2* mice. Specifically, it was found that in mutant mice the lateral, basolateral, central and medial nuclei of the amygdala are located in a more anterior position in the cortex when compared to controls (Kuemerle *et al.* 2007). Taken together, all these studies suggest that *En2*$^{-/-}$ mice are a suitable model for investigating the neurodevelopmental bases of ASD. It remains to be determined how the *En2* mutation can impact hippocampal excitability, and more generally, the structure and function of the forebrain. Indeed, the *En2* gene is commonly known for its crucial role in pattern formation of the midbrain and hindbrain regions. We will further investigate the effect of *En2* inactivation on the development of forebrain areas and their function in the adult brain, with particular attention to those neurodevelopmental, neuroanatomical and neurochemical features that are important for ASD. In addition, we plan to use *En2*$^{-/-}$ mice to test a behaviour-based therapeutic strategy for ASD.

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LIST OF ABBREVIATIONS

5-HT	*5-hydroxytryptamine,*
6-OHDA	*6-Hydroxydopamine*
8-OH-DPAT	*8-hydroxy-2-(di-n-propyl amino)tetralin*
A/P	*Antero-posterior*
ADHD	*Attention-deficit–hyperactivity disorder*
Amy	*Amygdala*
Bf	*Basal forebrain*
cc	*Corpus callosum*
CNS	*Central Nervous System*
Cpu	*Caudate-putamen*
Ctx	*Cerebral cortex*
D/V	*Dorso-ventral*
DA	*Dopamine*
DAT	*Dopamine transporter,*
DG	*Dentate gyrus*
Di	*Diencephalons*
DRN	*Dorsal Raphe Nucleus*
En	*Engrailed*
Ent	*Entorhinal cortex*
ES cell	*Embryonic stem cell*
FP	*Floorplate*
Fgf8	*Ffibroblast growth factor 8*
GABA	*Gamma-aminobutyric acid*
Gbx	*Gastrulation brain homeobox*
GPCR	*G protein-coupled receptors*
Hip	*Hippocampus*
HPLC	*High performance liquid chromatography*
HT	*Hypothalamus*

IEG	*Immediate early gene*
IHC	*Immunohistochemistry*
ISH	*In situ hybridization*
KA	*Kainic acid*
LGIC	*Ligand-gated ion channel*
Lmx	*LIM homeobox transcription factor*
MAO	*Monoamine Oxidase*
mDA	*Mesencephalic dopamine*
MHB	*Mid-hindbrain boundary*
MPTP	*1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine*
Mrp	*Mitochondrial ribosomal protein*
Ms	*Mesencephalon*
Msx	*Msh homeobox*
Mt	*Metencephalon*
nAcb	*Nucleus accumbens*
NeuN	*Neuronal Nuclei*
NPY	*Neuropeptide Y*
Ngn	*Neurogenin*
Nurr	*Nuclear receptor-related 1*
OM	*Oculomotor*
Otx	*Orthodenticle homologue*
P	*Prosencephalon*
Pax	*Paired box*
pCA	*para-chloroamphetamine*
pCPA	*para-chlorophenilalanine*
PCR	*Polymerase Chain reaction*
Pet	*Plasmacytoma expressed transcript 1*
Pitx	*Paired-like homeodomain transcription factor 3*
PV	*Parvalbumin*
Pyr	*Pyriform cortex*

r	*Rhombomeres*
RN	*Red nucleus*
SE	*Standard error*
Sept	*Septum*
SERT	*Serotonin transporter*
SD	*Standard deviation*
Shh	*Sonic hedgehog homolog*
SN	*Substantia nigra*
SOM	*Somatostatin*
SSRI	*Selective serotonin reuptake inhibitors*
TCA	*Tricyclic antidepressants*
Th	*Tyrosine hydroxylase*
Thal	*Thalamus*
TPH	*Tryptophan hydroxylase*
VMB	*Ventral midbrain*
VTA	*Ventral tegmental area*

ANNEXES

Brief Communications

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 F_1 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% $Na_2S_2O_5$, and 0.83 M $HClO_4$. 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 Millenium 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. 1*A*) (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. 1*A*). 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. 1*B*).

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. 2*A*, 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. 2*A*). 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. 2*A*). 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. 1*A*,*B*) (Borgkvist et al., 2006), increased 5-HT staining was detected in several areas including ventral midbrain, basal forebrain, cerebral cortex (Fig. 2*B*), and pons (raphe nuclei) (Fig. 2*B*,*C*) of saline-treated $En1^{Cre/+}$; $Otx2^{flox/flox}$ mutants, compared with saline-treated $Otx2^{flox/flox}$ controls. Treatment with

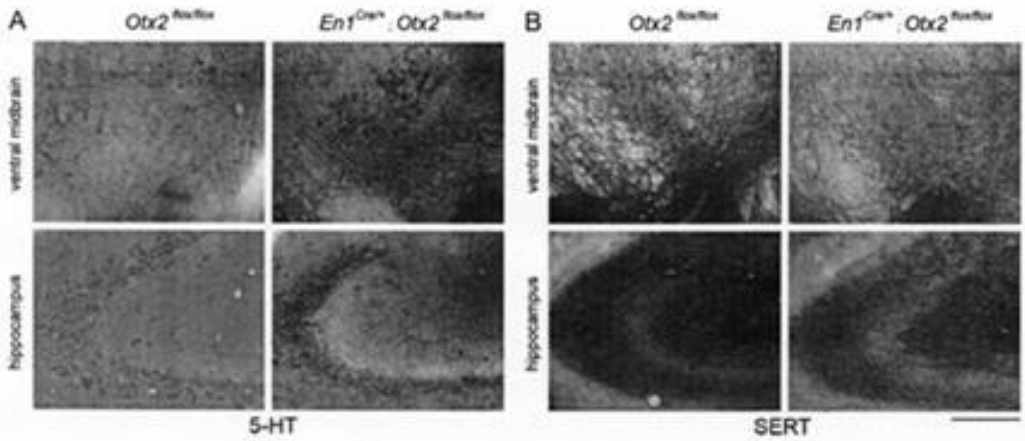

Figure 1. 5-HT is increased and SERT is decreased in the ventral midbrain and hippocampus of *Otx2* conditional mutant mice. ***A***, ***B***, Images show coronal sections through the ventral tegmental area of the midbrain (top) and CA3 region of the hippocampus (bottom) from $Otx2^{flox/flox}$ and $En1^{Cre/+}$;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

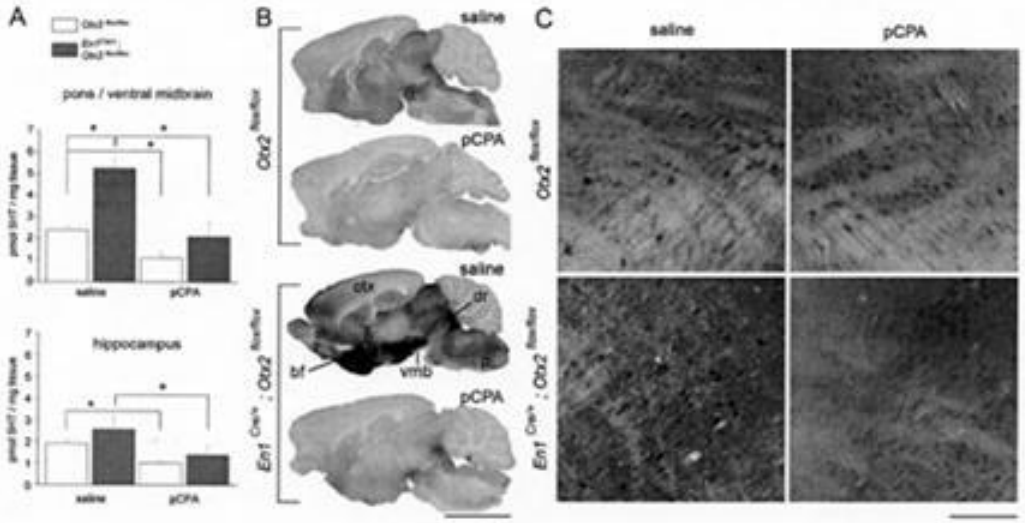

Figure 2. 5-HT depletion in control and *Otx2* conditional mutant mice. ***A***, HPLC determination of 5-HT content in the pons/ventral midbrain (top) and hippocampus (bottom) from $Otx2^{flox/flox}$ and $En1^{Cre/+}$; $Otx2^{flox/flox}$ mice, after a 3 d treatment with saline or pCPA. Data are reported as mean ± 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; ctx, 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

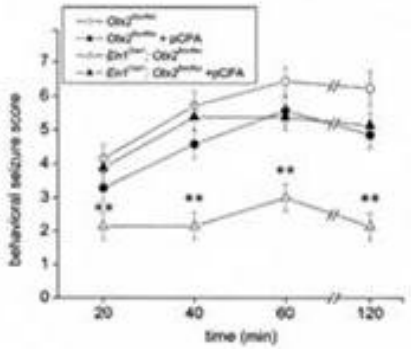

Figure 2. Resistance to KA-induced seizures in Otx2 conditional mutant mice is abolished by 5-HT depletion. Graph shows the progression of behavioral changes over a 2 h observation period after KA in control and *Otx2* conditional mutant mice, with or without pCPA pretreatment. Genotypes and treatments are as indicated. Data are reported as mean seizure scores ± 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-HT_3, and 5-HT_7 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 × 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 × 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 × 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

	$Otx2^{flox/flox}$	$En1^{Cre/+};Otx2^{flox/flox}$	$En1^{Cre/+};Otx2^{flox/flox}$ + pCPA	$Otx2^{flox/flox}$ + 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

[a]The number of animals with generalized seizures significantly differed between $En1^{Cre/+};Otx2^{flox/flox}$ and the other groups (χ^2 test, $p < 0.001$).

[b]Latency 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, $p = 0.362$).

[c]The value reported refers to the only animal that showed a generalized seizure.

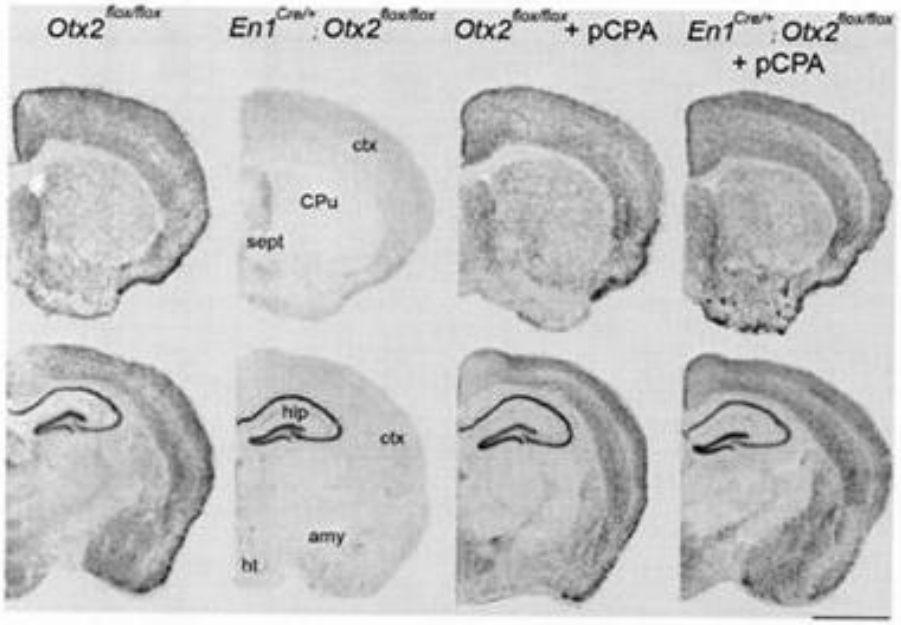

Figure 4. Effect of 5-HT depletion on c-*fos* mRNA expression in the brain of KA-treated control and *Otx2* conditional mutant mice. The panels show c-*fos* mRNA in situ hybridizations on coronal sections at the level of the caudate–putamen (top) and dorsal hippocampus (bottom) from representative control 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 D_1/D_2 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 $En1^{Cre/+}$; $Otx2^{flox/flox}$ mice would be expected to exacerbate seizure severity. In contrast, spontaneous or KA-induced seizures were never observed in $En1^{Cre/+}$; $Otx2^{flox/flox}$ 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 $En1^{Cre/+}$; $Otx2^{flox/flox}$ 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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Suppementary Results: quantification of immunohistochemistry experiments. A small number of 5-HT positive cells was detected in the ventral tegmental area (VTA) of $Otx2^{flox/flox}$ mice. These cells had a very small size and a rounded shape, suggesting that they were likely platelets (Brenner et al., 2007). Conversely, a higher number of 5-HT positive cells, clearly identifiable as neurons, was detected in the VTA of $En1^{Cre/+}$; $Otx2^{flox/flox}$ mice. Quantitative analysis confirmed the increased number of 5-HT cells in mutant mice (mean number of cells per counting box, ± SE: control, 7 ± 1; mutant, 14 ± 3; n = 36 counting boxes from 3 mice per genotype; t-test, $p<0.05$). Quantitative analysis also confirmed increased 5-HT staining in the CA3 area of *Otx2* conditional mutant mice, as compared to control mice (median value of 5-HT staining / background: control, 1.155; mutant, 1.491; n = 180 sampling windows from 3 mice per genotype; Mann-Whitney rank sum test, $p<0.001$). Quantitative analysis of 5-HT staining per cell in the dorsal raphe nucleus confirmed these findings. Mean values (± SE) of 5-HT staining per cell (normalized to background) were as follows: $Otx2^{flox/flox}$ saline, 1.90±0.02; $En1^{Cre/+}$; $Otx2^{flox/flox}$ saline, 2.22±0.02; $Otx2^{flox/flox}$ + pCPA, 1.65±0.03; En1Cre/+; $Otx2^{flox/flox}$ + pCPA, 1.63±0.03 (n = 150 cells from 5 animals per group). Statistical analysis confirmed that 5-HT levels were increased in saline-treated mutant mice, as compared to controls (one-way ANOVA, $p<0.001$; post hoc Holm-Sidak test, $p<0.001$) and that pCPA significantly decreased 5-HT staining in both genotypes (one-way ANOVA, $p<0.001$; post hoc Holm-Sidak test, $p<0.001$, control saline vs. control pCPA, and mutant saline vs. mutant pCPA). No difference was detected between control and mutant mice treated with pCPA (one-way ANOVA, $p>0.05$; post hoc Holm-Sidak test, $p>0.05$).

Suppementary Methods: quantitative analysis of immunohistochemistry experiments. Quantitative analyses of immuhistochemistry experiments were performed on digitized images (10x primary magnification) by using the Metamorph software. Three sections at the level of the ventral tegmental area (VTA), CA3 subfield (dorsal hippocampus) and dorsal raphe nucleus were taken from each animal (3-5 animals per genotype). To count 5-HT positive cells in the VTA, four squared counting boxes (75 mμ per side) were taken per section. 5-HT staining in the CA3 pyramidal layer was measured in 20 sampling windows (approximately the size of one cell) per section, and obtained values (grey levels) were divided by the background value measured in the callosum. 5-HT staining per cell in the dorsal raphe nucleus was measured in 10 cell bodies per section, and obtained values (grey levels) were divided by the background value measured in the callosum. Statistical analyses were performed by one-way ANOVA followed by appropriate post-hoc test, as indicated in the Supplementary Results.

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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′-agagagggcgcagttcttg-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,

taken at the level of the dorsal hippocampus, were analyzed using the Image J free software (http://rsb.info.nih.gov/ij/). For each section, signal intensity was measured in 10 different circular windows (area=0.01 mm^2) placed in layers 2–3 and 5–6 of the parietal/temporal cortex. Mean signal intensity was divided by the background labeling calculated in the corpus callosum. Statistical analysis was performed by Student's *t*-test.

Immunohistochemistry

For parvalbumin and somatostatin staining, three WT adult mice that never received KA were used. For neurodegeneration and NPY staining studies on KA-treated mice, five WT and seven $En2^{-/-}$ mice (see above) were killed 7 days after 20 mg/kg KA. Brains were fixed by immersion in 4% paraformaldehyde, cryoprotected in 30% sucrose/1× PBS and coronal sections (40 μm thick) were cut on a freezing microtome. Serial sections from the dorsal hippocampus were incubated overnight with appropriate antibodies as follows: anti-parvalbumin monoclonal (Sygma, Milan, Italy; 1:5000 dilution); anti-somatostatin polyclonal (Peninsula-Bachem, Weil am Rhein, Germany; 1:5000 dilution); anti-NeuN monoclonal (Chemicon, Temecula, CA, USA; 1:500 dilution) or anti-neuropeptide Y (NPY) polyclonal (Bachem, Weil am Rhein, Germany; 1:5000 dilution) antibodies. Signals were revealed with appropriate biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA, USA) followed by avidin–biotin–peroxidase complex (ABC kit, Vector Laboratories, Burlingame, CA, USA) and diaminobenzidine (DAB) reaction.

Neuronal damage was qualitatively assessed in CA1/CA3 areas of NeuN-stained sections from KA-treated mice, according to the following scale (Bozzi et al., 2000; Bozzi and Borrelli, 2002; Cilio et al., 2001): little damage, presence of scattered degenerated cells; mild damage, small areas with degenerated cells and/or tissue sclerosis; severe damage, extended areas of neuronal and fiber degeneration, accompanied by tissue sclerosis. Neurodegeneration was also confirmed by Nissl staining, performed on sections adjacent to those used for NeuN histochemistry. Nissl and NeuN staining confirmed cerebellar hypoplasia in $En2^{-/-}$ brains (not shown), according to previous studies (Joyner et al., 1991; Millen et al., 1994, 1995; Kuemerle et al., 1997).

To quantify NPY staining in the mossy fiber pathway of KA-treated mice, three sections through the dorsal hippocampus were analyzed per mouse. Images of mossy fiber pathway and of the overlying corpus callosum were digitized. Light intensity and microscope settings were optimized initially and then held constant. Care was taken to avoid saturation at either end of the pixel intensity range (0–255). For each section, NPY signal intensity was calculated in 10 boxes (40×40 μm) placed in mossy fiber pathway using the Image J free software (http://rsb.info.nih.gov/ij/). Mean signal intensity was divided by the background labeling calculated in the corpus callosum. Statistical analysis was performed by Mann–Whitney rank sum test.

RESULTS

En2 is expressed in the adult mouse hippocampus and cerebral cortex

Neuroanatomical and behavioural studies performed on adult $En2^{-/-}$ mice suggest that *En2* might be expressed also in anterior brain structures during adulthood (Cheh et al., 2006; Kuemerle et al., 2007). We therefore investigated *En2* mRNA expression in different brain areas of the adult mouse brain. To this purpose, we performed quantitative real-time RT-PCR experiments using the mitochondrial ribosomal L41 protein mRNA as a standard for quantification. As expected, L41 amplification gave comparable amplification curves from all brain areas analyzed (cerebellum, ventral midbrain, hippocampus, cerebral cortex; Fig. 1A). *En2* transcripts were detected at low but significant levels in the hippocampus and cerebral cortex; *En2* amplification curves from these two areas were indistinguishable (Fig 1B). According to previous studies (Joyner et al., 1991; Millen et al., 1994; Simon et al., 2001), *En2* mRNA was detected at higher levels in the ventral midbrain and cerebellum (Fig. 1B). Comparative quantification of real-time RT-PCR experiments showed that in hippocampus and cerebral cortex, *En2* mRNA was present about 100 times less than in cerebellum (Fig. 1C). In the adult hippocampus, *En2* mRNA levels were regulated by pathological hyperactivity. In animals that experienced generalized seizures following systemic administration of the glutamate agonist KA (20 mg/kg i.p.), *En2* mRNA levels were decreased by 30%, as compared to saline-treated controls (Fig. 1D).

$En2^{-/-}$ mice have a reduced expression of GABAergic markers in the hippocampus

Since *En2* is expressed in the adult hippocampus (Fig. 1B, C), we sought to investigate the presence of subtle neuroanatomical defects in this structure of $En2^{-/-}$ mice. Immunohistochemistry experiments with anti-parvalbumin and anti-somatostatin antibodies were performed to detect selected GABAergic interneuron populations (Matyas et al., 2004; Jinno and Kosaka, 2006) in the hippocampus of WT and $En2^{-/-}$ mice. In WT mice, parvalbumin revealed the typical staining around the cell bodies of pyramidal neurons of CA1 (not shown) and CA3 (Fig. 2) hippocampal subfields (see also Matyas et al., 2004), whereas somatostatin predominantly labeled stratum lacunosum moleculare and hilar interneurons (Fig. 2; see also Matyas et al., 2004). In $En2^{-/-}$ mice, staining for both parvalbumin and somatostatin was markedly reduced in CA3 pyramidal layer and stratum lacunosum moleculare, respectively (Fig. 2).

$En2^{-/-}$ mice display an increased susceptibility to KA seizures

Reduced expression of parvalbumin and somatostatin might indicate an altered GABAergic innervation (and, thus, excitability) of hippocampal circuitry in $En2^{-/-}$ mice. We therefore investigated seizure susceptibility in $En2^{-/-}$ mice. $En2^{-/-}$ mice never showed hyper-excitability or spontaneous seizures during standard housing. However, $En2^{-/-}$ mice presented a peculiar response when challenged with KA. The time course of the behavioral response of WT and $En2^{-/-}$ mice to KA (20 mg/kg) was evaluated over a period of 3 h after administration. In WT, this dose of KA generally resulted in the sole appearance of pre-convulsive behaviors at all time-points analyzed (Fig. 3). Only four out of eight WT mice displayed brief, isolated episodes of limbic motor seizures (rearing with forelimb clonus, stage 4), and never showed tonic–clonic (stage 6) seizures. The same KA dose in $En2^{-/-}$ mice elicited clear signs of focal epilepsy (head bobbing) within the first 20 min, rapidly culminating in stage 4 limbic motor seizures. Latency to the first stage 4 seizure did not differ

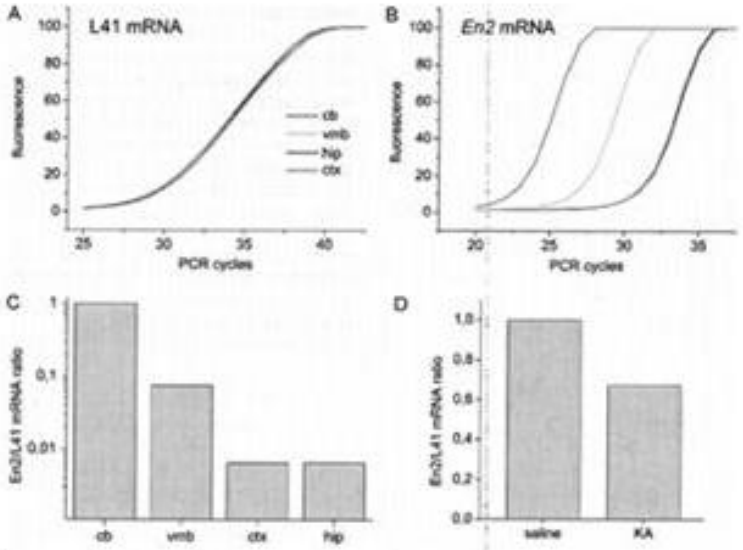

Fig. 1. *En2 mRNA* is expressed in adult mouse hip and ctx and is regulated by seizure activity. (A, B) Real-time RT-PCR amplification profiles of mitochondrial ribosomal protein L41 (A) and *En2* (B) mRNAs from the cb (blue line), vmb (green line), hip (black line) and ctx (red line) of WT adult mice. The graphs report the appearance of fluorescence in PCR amplicons as a function of the number of PCR cycles. (C, D) Graphs report the quantification of real-time RT-PCR experiments. In (C), values are expressed as *En2* mRNA/L41 mRNA comparative quantitation ratios (average values of three technical replicates) in different adult brain areas, normalized to cb. In (D), values are expressed as *En2* mRNA/L41 mRNA comparative quantitation ratios (average values of three technical replicates), from the hip of KA-treated adult mice (3 h post KA, 20 mg/kg i.p.), normalized to saline-treated controls. Abbreviations: cb, cerebellum; ctx, cerebral cortex; hip, hippocampus; vmb, ventral midbrain. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

from that observed in WT mice (Table 1). In sharp contrast with WT, the majority (7 out of 12) of $En2^{-/-}$ mice displayed severe tonic–clonic seizures (Table 1). $En2^{-/-}$ mice showed generalized stage 4–6 seizures for about 2 h (40–160 min; Fig. 3). Statistical analysis performed by two-way repeated measures ANOVA revealed a significant effect of genotype ($F_{1,136}$= 7.522, P= 0.014). Multiple com-

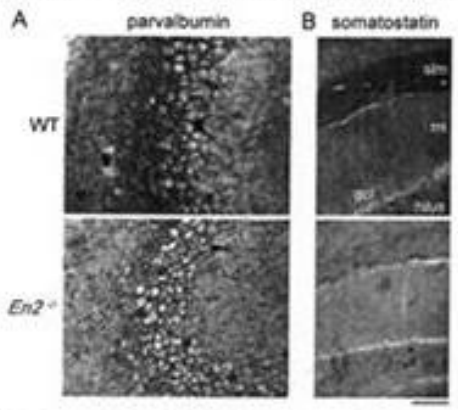

Fig. 2. Downregulation of GABAergic markers in the hippocampus of $En2^{-/-}$ mice. (A) Representative parvalbumin staining in the CA3 pyramidal cell layer of WT and $En2^{-/-}$ mice. (B) Representative somatostatin staining in the slm and hilus of WT and $En2^{-/-}$ mice. Abbreviations: gcl, granule cell layer; mi, molecular layer; slm, stratum lacunosum moleculare. Scale bar = 50 μm.

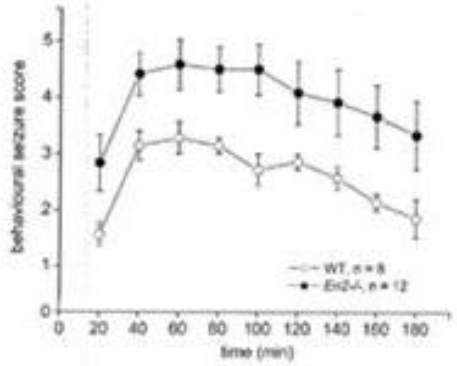

Fig. 3. Increased susceptibility to KA-induced seizures in $En2^{-/-}$ mice. Progression of behavioral changes after systemic KA administration (20 mg/kg i.p.) in WT and $En2^{-/-}$ over a 3 h observation period. Data are mean seizure scores ± SE.

Table 2. Cell damage in hippocampal CA1/CA3 pyramidal layers of KA-treated WT and *En2*$^{-/-}$ mice

Brain area/genotype	Degree of cell damage (no. of animals)			
	None	Little	Mild	Severe
CA1				
WT (n=5)	5	0	0	0
En2$^{-/-}$ (n=7)	3	2	1	1
CA3				
WT (n=5)	3	1	1	0
En2$^{-/-}$ (n=7)	3	2	0	2

Brain damage was evaluated in NeuN-stained sections, 7 days after KA, according to the scale described in Experimental Methods.

mossy fibers of dentate gyrus in all *En2*$^{-/-}$ mice (Fig. 6B), whereas no such labeling was detected in WT animals (Fig. 6A). Quantification of NPY staining in the mossy fiber pathway from WT and *En2*$^{-/-}$ mice confirmed these findings (Fig. 6C). Saline-treated mice of both genotypes did not show any sign of hippocampal histopathology (not shown).

DISCUSSION

By using behavioral, gene expression and neuroanatomical analyses, we provided evidence that *En2*$^{-/-}$ mice have an increased susceptibility to KA-induced seizures and long-term histopathology. *En2*$^{-/-}$ mice displayed more severe and prolonged generalized seizures as compared to WT mice. The occurrence of generalized seizures in *En2*$^{-/-}$ mice was accompanied by the widespread mRNA induction of the IEGs c-fos and c-jun, as well as CA1 cell loss and NPY upregulation in mossy fibers.

Systemic KA administration has been widely used to study the susceptibility to acute seizures and seizure-induced long-term histopathology in inbred and mutant mouse strains. In the present study, control and *En2*$^{-/-}$ mice of a mixed 129/Sv×C57BL/6 genetic background were used. According to our previous studies (Bozzi et al., 2000), WT mice displayed a very mild response to 20 mg/kg KA, never showing continuous generalized epileptic activity (stage 5 seizures) or tonic–clonic seizures (stage 6) after KA. Conversely, *En2*$^{-/-}$ mice displayed high susceptibility to seizures induced by the same dose of KA, indicating a seizure-promoting effect of *En2* inactivation in the mixed 129/Sv×C57BL/6 background. Increased seizure susceptibility in *En2*$^{-/-}$ mice was also confirmed by gene expression studies. A precise correlation exists between the occurrence of generalized seizures and the induction pattern of the IEGs c-*fos* and c-*jun* following KA. Pre-convulsive behaviors (stages 1–3 of the Racine's scale) induce IEGs mainly in the hippocampus and other limbic areas (hippocampus, amygdala, entorhinal and pyriform cortices) whereas continuous generalized seizures (stages 4–6) result in a widespread expression in several brain areas (Willoughby et al., 1997; Bozzi et al., 2000). Accordingly, WT mice experienced no or very brief generalized seizures after KA, showing IEGs mRNA induction restricted to the hippocampus and other limbic areas. Conversely, *En2*$^{-/-}$ mice showed long-lasting generalized seizures and widespread induction of IEGs mRNA throughout the brain. It is important to point out that spontaneous seizures never occurred in naive *En2*$^{-/-}$ mice (data not shown), indicating that increased susceptibility of *En2*$^{-/-}$ mice is evident only in response to a potent seizure-promoting stimulus, such as KA.

KA-induced seizures were also followed by long-term histopathological changes in the hippocampus of *En2*$^{-/-}$ mice. In mice, the occurrence of brain damage following KA-evoked seizures strongly depends on the genetic background; certain mouse strains, such as inbred C57BL/6 or mixed 129/Sv×C57BL/6, are resistant to KA-induced cell death (Schauwecker and Steward, 1997; Schauwecker et al., 2000; McLin and Steward, 2006). In keeping with these findings, our WT mice never showed cell damage in the CA1 pyramidal layer and only occasionally presented little or mild damage in the CA3. A marked degeneration was instead observed in all *En2*$^{-/-}$ mice analyzed in the CA1 and, to a lesser extent, in the CA3. This variability in the degree of brain damage following systemic KA administration, that we observed in both WT and *En2*$^{-/-}$ mice, is in agreement with previous studies (Schauwecker and Steward, 1997; Bozzi et al., 2000; McLin and Steward, 2006). Most importantly, *En2*$^{-/-}$ but not WT mice treated with KA displayed NPY upregulation in the mossy fiber pathway (dentate gyrus to CA3). This pattern of NPY upregulation is generally considered a reliable marker of long-term, post-seizure synaptic rearrangements, and is thought to be

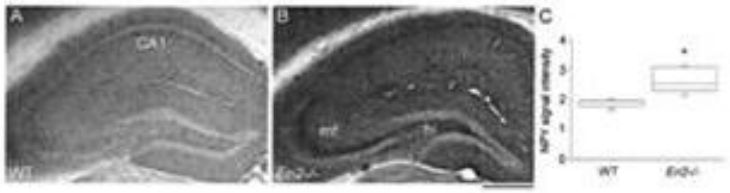

Fig. 6. NPY upregulation in the *mf* pathway of KA-treated *En2*$^{-/-}$ mice. (A, B) Representative NPY staining in the hippocampus of WT (A) and *En2*$^{-/-}$ (B) mice, 7 days after KA. The almost complete loss of CA1 is also visible in (B). Abbreviations: CA1, pyramidal cell layer; (H) hilus; *mf*, mossy fibers. Scale bar = 500 μm. (C) Quantification of NPY staining intensity in the *mf* of WT and *En2*$^{-/-}$ mice. Each box chart summarizes the distribution of the NPY signal-to-background ratio (intensity of NPY label divided by the background staining) for all hippocampal sections in each group. The horizontal lines in the box denote the 25th, 50th, and 75th percentile values. The error bars denote the 5th and 95th percentile values. * $P<0.05$ (Mann–Whitney rank sum test).

indicative of an acquired hyper-excitability of the hippocampus following seizures (Morimoto et al., 2004; Nadler et al., 2007; Dudek and Sutula, 2007; Sperk et al., 2007; Sutula and Dudek, 2007). Moreover, as observed for pyramidal cell loss, rearrangements in mossy fibers do not occur following KA in mouse strains derived from C57BL/6 (Schauwecker et al., 2000). Our data suggest that inactivation of the *En2* gene also results in long-term anatomical modifications and, likely, hyper-excitability of hippocampal circuitry in response to KA seizures.

It remains to be determined how the *En2* mutation can impact hippocampal excitability. Indeed, the *En2* gene is commonly known for its crucial role in pattern formation of the midbrain and hindbrain regions (see the introduction). Our data show that in the adult mouse brain, *En2* is expressed in the hippocampus and cerebral cortex, two regions crucially involved in seizure generation and spread. These results confirm and expand non-quantitative RT-PCR expression data previously published on-line in the mouse genome informatics (MGI) database (http://www.informatics.jax.org). Moreover, we showed for the first time that the expression of the *En2* gene in the adult hippocampus is downregulated by seizure activity. This expression profile indicates that *En2* may be also involved in the functioning of adult brain areas and in their response to seizures.

The reduced staining of parvalbumin and somatostatin in the hippocampus of $En2^{-/-}$ mice suggests a reduced GABAergic innervation onto CA3 and CA1 pyramidal neurons, that might explain the hyper-excitability observed in these mutants. The present study, along with recent findings from other authors (Kuemerle et al., 2007), suggests that *En2* is implicated also in the development of telencephalic structures. Kuemerle et al. (2007) reported an anterior shift of the amygdala in $En2^{-/-}$ mice. Specifically, it was found that in mutant mice the lateral, basolateral, central and medial nuclei of the amygdala are located in a more anterior position in the cortex when compared to controls. These anatomical alterations, together with those detected in the hippocampus, might contribute to alter circuitry and excitability of the limbic system in $En2^{-/-}$ mice. EN2 secretion from posterior structures to anterior target areas could explain these effects (Joliot et al., 1998; Brunet et al., 2005; Sonnier et al., 2007). Further studies might be aimed at determining whether EN2 released from ventral midbrain projections can influence excitability of more anterior limbic structures.

Our results strengthen the notion that $En2^{-/-}$ mice can be used as a model to study at least some of the multiple pathological aspects of ASD. In particular, we propose to use these mutants to further investigate the role of altered circuitry/excitability of the limbic system in ASD and, ultimately, the relationship between epilepsy and ASD. Indeed, morphological abnormalities (likely of developmental origin) have been detected in the limbic system of autistic patients, that might underlie increased excitability. Classical anatomical studies showed increased cell density and reduced cell size in limbic structures including hippocampus, subiculum and amygdala. Reduced dendritic arborizations were also observed in the same structures from autistic patients (Kemper and Bauman, 1993; Palmen et al., 2004; Bauman and Kemper, 2005). It will be also interesting to exploit *En2* mutant mice to understand how the inactivation of a gene involved in midbrain/hindbrain patterning can affect the embryonic development (and adult functioning) of more anterior, telencephalic structures.

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Chapter 15

Developmental basis of epilepsy and seizure susceptibility: role of *Otx* genes

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Summary

The embryonic development of the central nervous system (CNS) requires an orchestrated series of events tightly regulating the patterning and regionalization of the neural tube, as well as the proliferation, survival, and differentiation of distinct neuronal populations. All these events are controlled by cascades of activation of transcription factors which regulate the expression of specific subsets of genes in restricted regions and neuronal populations of the developing CNS. Among these transcription factors, homeobox-containing proteins play a crucial role, and altered expression of these factors can affect both embryonic and adult CNS functions. Indeed, mutations of homeobox genes have been associated with human syndromes characterized by the occurrence of epileptic seizures. Molecular genetic studies carried out in the mouse also demonstrated that inactivation of homeobox-containing genes can have a marked impact on specific stages of brain development, leading in some cases to altered seizure susceptibility in adult life. In this chapter, we focus on *Otx* genes, and on the effects of their genetic inactivation on seizure susceptibility.

Introduction

A large series of studies undertaken in the past three decades showed that homeobox-containing transcription factors crucially control all stages of the embryonic development of the vertebrate central nervous system (CNS). The induction, specification, and regionalization of the brain, as well as the proliferation, survival, and differentiation of distinct neuronal populations, depend on the tightly regulated expression of homeobox genes. The activity of these ultimately regulates the expression of specific genes controlling the identity of restricted regions and neuronal populations in the developing CNS (Rubenstein & Puelles, 1994). Altered expression and function of homeobox genes can markedly affect embryonic brain development, leading to severe postnatal neurological dysfunction, including epilepsy.

Homeobox genes involved in human epileptic disorders

The developmental basis of epilepsy is largely unknown. However, it is conceivable that altered function of genes controlling the specification of brain areas, neuronal identity, and circuit formation can lead to increased seizure susceptibility and epilepsy. For example, abnormal expression and function of genes involved in brain development might lead to altered differentiation of selected neuronal populations and improper shaping of neuronal circuitry, thus resulting in imbalance between excitation and inhibition in the postnatal brain. It is obvious that many mutations of developmental genes may be lethal, as such genes control early stages of embryonic development. Nevertheless, selective inactivation of developmental gene functions in restricted brain areas or neuronal populations might be compatible with adult life, though compromising normal brain function. According to this view, in recent years several laboratories have tried to identify mutations of developmental genes associated with epileptic disorders. Many of these studies focused on homeobox genes, owing to their prominent role in controlling CNS development. A summary of the major findings, obtained by browsing the Online Mendelian Inheritance in Man (OMIM) database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=omim), is given in Table 1.

Table 1. Human and mouse homeobox genes related to seizure disorders

Homeobox gene	OMIM N	Human disease / phenotype	Animal model (reference)
Zinc finger E-box binding homeobox 2 (ZEB2/ZFHX1B)	605802	Hirschsprung disease – variants with mental retardation, delayed motor development, epilepsy, and neurocristopathies at cephalic, cardiac, and vagal level	*Zfhx1b*$^{-/-}$ mice (Van de Putte *et al.*, 2003)
Aristaless-related homeobox, X-linked (ARX)	300382	X-linked mental retardation and epilepsy (X-linked, early onset epileptic encephalopathy 1: subgroup of West syndrome, with infantile spasms, EEG pattern of hypsarrhythmia and mental retardation). ARX mutations can also cause Partington syndrome (OMIM No. 309510) and lissencephaly (OMIM No. 300215)	*Arx*$^{-/-}$ mice (Kitamura *et al.*, 2002) (Colombo *et al.*, 2007)
Homologous of Drosophila empty spiracles 2 (EMX2)	600035	Schizencephaly, in some cases associated to partial epilepsy (Brunelli *et al.*, 1996; recently not confirmed, Merello *et al.*, 2008)	*Emx2*$^{-/-}$ mice (Mallamaci *et al.*, 2000a,b)
Homologue of Drosophila muscle segment homeobox 2 (MSX2)	604757	Craniosynostosis (skull malformations with severe headache and seizure disorder)	*Msx2* transgenic mice (Liu *et al.*, 1995)
Distal-less homeobox 1 (DLX1)	600029	Not known	*Dlx1*$^{-/-}$ mice (Cobos *et al.*, 2005)
Homologous of Drosophila ortidenticle 1 (OTX1)	600036	Not known	*Otx1*$^{-/-}$ mice (Acampora *et al.*, 1996)
Homologous of Drosophila ortidenticle 2 (OTX2)	600037	Syndromic microphthalmia with severe learning difficulties, and seizure disorder	See text

The table reports the results of searching the Online Mendelian Inheritance in Man (OMIM) database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=omim) by using 'homeobox AND epilepsy' and 'homeobox AND seizure' as keywords. Search results are updated at 28 July 2008. For an extensive review on the genetic bases of brain malformations and epilepsy see Guerrini & Marini, 2006.

The most consistent and convincing example is represented by the *Aristaless*-related homeobox *(Arx)*, mutations of which have been associated with a series of early onset X-linked syndromes characterized by mental retardation and epilepsy (OMIM No. 300382) (Bienvenu *et al.*, 2002; Kitamura *et al.*, 2002; Stromme *et al.*, 2002). The various forms of the disease represent a subgroup of West syndrome, characterized by infantile spasms, EEG abnormalities, mental retardation, and abnormal genitalia. *Arx* mutations have also been associated with Partington disease and lissencephaly (Sherr, 2003) (see Table 1 for OMIM numbers). Mouse molecular genetic studies were undertaken to develop an animal model of the human disease. The first study by Kitamura *et al.* (2002) demonstrated that a loss-of-function mutation of *Arx* in the mouse resulted in microcephaly. Moreover, *Arx* knockout mice displayed aberrant differentiation of γ-aminobutyric acid (GABA)-producing interneurons (GABAergic interneurons) in the basal forebrain and neocortex, reproducing some of the prominent clinical features of X-linked lissencephaly with abnormal genitalia (XLAG) in humans. More recently, Colombo *et al.* (2007) expanded these findings by showing that *Arx* mutant mice present marked migration defects of GABAergic interneurons, are lacking a large fraction of cholinergic neurons, and have abnormal thalamo-cortical projections.

Mutations of other homeobox-containing genes – such as *Zeb2*, *Msx2*, and *Emx2* – have also been associated with complex syndromes characterized by the occurrence of epileptic seizures (Table 1). In particular, the role of *Emx2* has been debated, as the association of mutations of this gene with schizencephaly (Brunelli *et al.*, 1996) has not been confirmed in a recent study (Merello *et al.*, 2008). Finally, studies carried out in the mouse suggested that *Dlx* and *Otx* genes might be causally linked to developmentally-related epilepsies, though mutations in these genes have never been found in association with human seizure disorders. Indeed, genetic inactivation of *Dlx1* in the mouse results in reduced numbers of GABAergic interneurons during brain development, leading, in adult animals, to reduced inhibition in the cerebral cortex and subsequent epilepsy (Cobos *et al.*, 2005). Loss-of-function mutations of *Otx1* and *Otx2* genes have not been described in human epilepsies. The only exception is represented by a single case of syndromic microphthalmia with learning deficit and a seizure disorder that was associated with a mutation in exon 3 of the *Otx2* gene, resulting in a truncated C-terminal domain of the protein (Ragge *et al.*, 2005) (Table 1). Nevertheless, a series of studies has shown that *Otx* gene inactivation can alter seizure susceptibility in the mouse, suggesting that these genes may play an important role in the development of the brain structures involved in the genesis of epilepsy (see below and Table 2).

The role of *Otx* genes in seizure susceptibility

The vertebrate homologues of the *Drosophila orthodenticle (otd)* gene, *Otx1* and *Otx2*, code for transcription factors containing a bicoid-like DNA-binding homeodomain (Finkelstein & Boncinelli, 1994; Simeone, 1998). *Otx* genes are specifically expressed in the developing brain: *Otx2* is expressed in all dorsal and most ventral regions of the telencephalon, diencephalon and mesencephalon, whereas the expression domain of *Otx1* is similar to that of *Otx2*, but contained within it (Simeone *et al.*, 1992).

Homologous recombination of *Otx1* in the mouse leads to epilepsy (Acampora *et al.*, 1996). *Otx1*$^{-/-}$ mice display high speed turning behaviour accompanied by electrographic (EEG) seizures in the hippocampus and cerebral cortex. Anatomically, *Otx1*$^{-/-}$ brains have a marked reduction in thickness and cell number in the temporal and perirhinal areas of the cerebral cortex, a disorganization of cortical layering in the same areas, and a shrinkage of the hippocampal formation (Acampora *et al.*, 1996). More detailed morphological and functional studies showed that inactivation of *Otx1*

results in selective loss of layer V pyramidal neurons, ectopic pyramidal cells in layers II–III, and altered expression of GABAergic markers (parvalbumin and glutamic acid decarboxylase) in the cerebral cortex (Avanzini *et al.*, 2000, Cipelletti *et al.*, 2002). These anatomical abnormalities may be the primary cause of hyperexcitability and seizure occurrence in *Otx1*$^{-/-}$ mice. Indeed, increased excitatory NMDA-mediated neurotransmission can be detected in the cerebral cortex of *Otx1* mutants (Sancini *et al.*, 2001) (Table 2). Importantly, introduction of the *Drosophila otd* or the human *Otx2* gene into the *Otx1* locus can completely rescue the epileptic phenotype of *Otx1* mutants, thus demonstrating that *Otx* and *otd* gene functions are highly evolutionarily conserved at a functional level (Acampora *et al.*, 1998; Acampora *et al.*, 1999).

Table 2. Phenotypes of *Otx* mutant mouse strains, with relevance to seizure susceptibility

Mutant strain	Homologous recombination strategy	Phenotype	Reference
Otx1$^{-/-}$	*Otx1* total knockout	Cortical abnormalities, generalized epilepsy with hippocampal and cortical EEG alterations	Acampora *et al.*, 1996
		Selective loss of layer V pyramidal neurons, ectopic pyramidal cells in layers II–III, altered GABAergic markers (parvalbumin and glutamic acid decarboxylase) in somatosensory cortex	Avanzini *et al.*, 2000 Cipelletti *et al.*, 2002
		Increased NMDA-mediated polysynaptic excitation in the cerebral cortex	Sancini *et al.*, 2001
otd/*otd*^{1}	Drosophila orthodenticle (*otd*) gene expressed in the *Otx1* locus	No cortical abnormalities, no epilepsy/EEG seizures	Acampora *et al.*, 1998
hOtx2^{1}/*hOtx2*^{1}	Human *Otx2* gene expressed in the *Otx1* locus	Reduced cortical abnormalities, no epilepsy/EEG seizures	Acampora *et al.*, 1999
Otx1$^{Cre/+}$; *Otx2*$^{flox/-}$	*Otx2* inactivated in *Otx1* expression domain	Glutamate to GABA switch of glutamatergic progenitors in the thalamus; die at birth	Puelles *et al.*, 2003 Puelles *et al.*, 2006
En1$^{Cre/+}$; *Otx2*$^{flox/flox}$	*Otx2* inactivated in *Engrailed1* expression domain (dopaminergic progenitors)	Dopamine to serotonin switch; resistance to kainic acid seizure due to serotonin hyper-innervation	Puelles *et al.*, 2004 Borgkvist *et al.*, 2006 Tripathi *et al.*, 2008

The table reports the phenotype of different mouse strains in which *Otx* genes were inactivated by homologous recombination. Only the phenotypes that are relevant to seizure disorders are listed; the reader is referred to the quoted original articles for an extensive description of mutant mice.

Loss-of-function mutation of *Otx2* in mice is lethal, homozygous mutant embryos being characterized by the complete absence of forebrain and midbrain regions (Acampora *et al*, 1995). The headless phenotype of *Otx2*$^{-/-}$ embryos might be due to the very early expression and function of *Otx2* in developing embryos (already present at the time of gastrulation, well before the beginning of neural tube formation (reviewed in Acampora *et al.*, 1999). The extreme phenotype of *Otx2*$^{-/-}$ embryos does not allow a detailed investigation of *Otx2* function in the developing brain. However, this has been successfully achieved by selectively inactivating *Otx2* in restricted brain regions during development through a 'conditional knockout' strategy (reviewed in: Lewandoski, 2001; Gavériaux-Ruff & Kieffer, 2007), which is schematically illustrated in Fig. 1A.

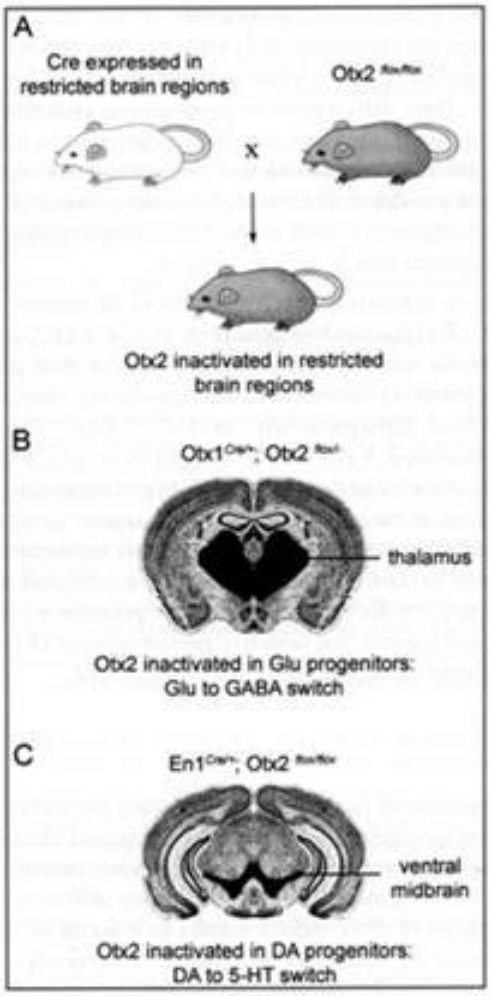

Fig. 1. Otx2 inactivation in restricted brain areas. (A) General strategy followed to inactivate Otx2 in restricted brain areas during mouse development. This strategy is based on the use of two different parental strains: a transgenic mouse line expressing the Cre recombinase under the control of a tissue-specific promoter, and a mutant mouse ($Otx2^{flox/flox}$) in which Otx2 is flanked by brief loxP sequences, which are recognized by the Cre recombinase. $Otx2^{flox/flox}$ mice are normal, since loxP sequences do not interfere with Otx2 function. When this line is crossed with the tissue-specific Cre transgene, the Cre binds to loxP sites and excides the Otx2 gene, resulting in Otx2 knockout only in the cells that express Cre. (B) Selective inactivation of Otx2 in the Otx1 expression domain leads to glutamate (Glu) to GABA switch in glutamatergic progenitors of the thalamus (in black). (C) Selective inactivation of Otx2 in Engrailed1 expression domain leads to dopamine (DA) to serotonin (5-HT) switch in DAergic progenitors of the ventral midbrain (in black). See text for details and references. Brain images in B and C have been freely downloaded from the Allen Brain Atlas website (http://mouse.brain-map.org) and modified by using Adobe Photoshop software.

In an initial study, *Otx2* was inactivated in the more restricted expression domain of *Otx1*. $Otx1^{Cre/+}$;$Otx2^{flox/-}$ mice die at birth and show abnormal development of the midbrain (Puelles *et al.*, 2003). Interestingly, *Otx2* inactivation in the *Otx1* expression domain leads to a glutamate

(Glu) to GABA switch in glutamatergic progenitors of the thalamus. During normal brain development, *Otx2* prevents the expression of GABAergic markers by repressing the transcription factor *Mash1 (mammalian achaete-schute homologue)*, and *Otx2* inactivation in neurons expressing *Otx1* modifies their differentiation programme, switching on GABAergic genes (Puelles *et al.*, 2006). This study provides the first evidence for a crucial role of *Otx2* in the molecular mechanism regulating the identity and fate of glutamatergic precursors in the thalamus. It would be important to determine whether the same molecular cascades also take place in other brain areas expressing *Otx2* – such as the developing cerebral cortex and hippocampus – which play a more prominent role in epileptogenesis.

A second strategy aimed at selectively inactivating Otx2 in neurons of the ventral midbrain expressing the *Engrailed1 (En1)* homeobox gene (Fig. 1C). In $En1^{cre/+}$;$Otx2^{flox/flox}$ mice, midbrain dopamine neurons are greatly reduced in number and most of their precursors undergo neurotransmitter fate switch, generating serotonin (5-HT)-producing neurons (Puelles *et al.*, 2004). This alteration is maintained throughout life, as $En1^{cre/+}$;$Otx2^{flox/flox}$ adult mice still display reduced dopamine and increased 5-HT levels (Borgkvist *et al.*, 2006), though not showing gross neurological defects. As a consequence of 5-HT hyperinnervation, $En1^{cre/+}$;$Otx2^{flox/flox}$ mice were resistant to generalized seizures induced by the glutamate agonist kainic-acid (KA). Prolonged pretreatment of mutant mice with the 5-HT synthesis inhibitor para-chlorophenylalanine reduced 5-HT brain content to control levels and fully re-established KA-seizure susceptibility (Tripathi *et al.*, 2008). These results clearly confirm the prominent role of *Otx2* in neurotransmitter fate specification, and suggest that selective perturbation of *Otx2* expression in the developing brain may alter seizure susceptibility in the adult animal.

Conclusions

Altered expression and function of homeobox genes during the embryonic development of the brain may lead to abnormal specification of brain areas, neuronal identity, and circuit formation, ultimately resulting in impaired neurological function. Studies carried out in the mouse suggest that inactivation of the two *Otx* genes, *Otx1* and *Otx2*, may differentially alter seizure susceptibility. Complete inactivation of *Otx1* indeed results in a series of anatomical defects of the cerebral cortex accompanied by spontaneous seizures. Conversely, selective inactivation of *Otx2* may result in suppression of experimentally-induced seizures. Further studies might address the question of whether these molecular mechanisms can be detected in the healthy and epileptic human brain.

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Developmental basis of seizure susceptibility: a focus on dopaminergic and serotonergic systems

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ABSTRACT

The embryonic development of the Central Nervous System (CNS) requires an orchestrated series of events tightly regulating the patterning and regionalization of the neural tube, as well as the proliferation, survival and differentiation of distinct neuronal populations. All these events are controlled by cascades of activation of transcription factors that regulate the expression of specific subsets of genes in restricted regions and neuronal populations of the developing CNS. Among these transcription factors, homeobox-containing proteins play a crucial role, and altered expression of these factors can impact embryonic as well as adult CNS functions. In particular, homeobox-containing genes have been described to crucially regulate differentiation of dopaminergic and serotonergic neurons during brain development. Dopaminergic and serotonergic neurons, respectively located in midbrain and hindbrain regions, diffusely innervate several forebrain areas, contributing to regulate several physiological functions including brain excitability. Classical pharmacological studies clearly showed that both dopamine and serotonin markedly regulate seizure susceptibility through specific receptor pathways. Our recent studies, performed on classical and conditional knockout mouse lines, demonstrate that altered embryonic development of dopaminergic and serotonergic neurons results in altered seizure susceptibility in the adult life. Here we will review our major findings, in light of other studies recently published by other groups.

KEYWORDS
Dopamine, serotonin, epilepsy, limbic system, knockout mouse

INTRODUCTION

Epilepsy is one of the most common neurological disorders, affecting about 1% of human population worldwide. The disease is characterized by the repeated occurrence of sudden, transitory and localized bursts of electrical activity, known as seizures. Seizures may arise in both cortical and subcortical areas, and depending on the brain area that is affected may result in episodes of motor, sensory, autonomic and psychic origin. Genetic, traumatic and developmental factors have been clearly implicated in the genesis of epilepsy.

The developmental bases of epilepsy are largely unknown. Altered function of genes controlling specification of brain areas, neuronal identity and circuit formation may certainly lead to altered seizure susceptibility and epilepsy. For example, abnormal expression and function of genes involved in brain development might lead to altered differentiation of selected neuronal populations and improper shaping of neuronal circuitry, thus resulting in imbalance between excitation and inhibition in the postnatal brain.

A vast series of clinical and experimental studies clearly demonstrate that all major neurotransmitter systems are involved in epileptogenesis, including dopamine (DA) and serotonin (5-hydroxytryptamine, 5-HT) [1, 2]. Classical pharmacological studies clearly showed that both DA and 5-HT may have potent anti-convulsant effects, acting through specific receptor pathways. However, little is known about the impact of an altered embryonic development of dopaminergic and serotonergic neurons onto seizure susceptibility in the adult life. Here we will review our recent findings resulting from the study of classical and conditional knockout mouse lines with an altered development of DA and 5-HT systems.

ROLE OF DA AND 5-HT IN EPILEPSY

The role of DA and 5-HT in the genesis and control of seizures has been extensively reviewed in previous studies [1-4], to which the reader is referred for a detailed description of the experimental data. Here we will briefly summarize the major findings in this field.

DA and epilepsy. DA has long been postulated to have an anti-epileptic action. The anti-convulsant properties of apomorphine (a prototypic DA agonist) were first described more than one century ago. Seizure inhibition has been also observed in patients administered amphetamines or antiparkinsonian drugs such as pergolide and bromocriptine, which all stimulate dopaminergic transmission [2].

The use of dopaminergic ligands specific for the different subclasses of DA receptors allowed to demonstrate that DA has an anti-epileptic action also in a wide variety of animal models [2, 5]. In particular, these studies illustrated the opposite actions of D1-like and D2-like receptors in the regulation of seizure activity. The physiological balance of DA activity at these two different receptors would be crucial for determining the response to seizure-promoting stimuli: activation of D1-like receptors is generally pro-convulsant, whereas D2-like receptor stimulation can block seizures. More recently, studies performed on different dopamine receptor knockout mouse lines confirmed these findings [3, 6-8]. The limbic system is crucially involved in the dopaminergic control of epileptic seizures. Indeed, limbic areas of the brain receive dopaminergic innervation [9] and express different types of DA receptors [10].

5-HT and epilepsy. The idea that there may be a link between 5-HT and seizure inhibition was first suggested as early as 1957 [11]. In this study, a series of anticonvulsants, including phenytoin, were shown to elevate brain 5-HT levels. In recent years, there has been increasing evidence that serotonergic neurotransmission can modulate seizures in a wide variety of experimental models. It is now generally accepted that drugs elevating extracellular 5-HT levels (such as 5-hydroxytryptophan or selective serotonin reuptake inhibitors, SSRI) exert a powerful antiepileptic action against both focal (limbic) and generalized seizures [12-14]. 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 by para-chloroamphetamine (PCA, a selective neurotoxin for 5-HT neurons) or para-chlorophenilalanine (pCPA, an inhibitor of 5-HT synthesis) can lower seizure threshold, increasing the severity of limbic status epilepticus [1, 15].

Fourteen mammalian 5-HT receptor subtypes are currently recognized, and these have been classified into seven receptor families on the basis of their structural, functional and, to some extent, pharmacological characteristics [16, 17]. Among these receptors, the 5-HT_{1A}, 5-HT_{2C}, 5-HT_3 and 5-HT_7 subtypes, which are all expressed in epileptogenic brain areas (mainly, cerebral cortex and/or hippocampus), are the most relevant in epilepsy [1]. For example, administration of 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT, a specific 5-HT_{1A} agonist) is able to reduce experimentally induced seizures in rats [18], whereas increased lethality after seizures is observed in mice with targeted inactivation of the 5-HT_{1A} gene [19]. Mice lacking 5-HT_{2C} receptors also develop epilepsy [20, 21].

Taken together, all these studies clearly demonstrate that pharmacological and genetic manipulation of DA and 5-HT levels can markedly affect seizure origin and spread; conversely, little is known about the impact of an altered embryonic development of DA and 5-HT neurons onto seizure susceptibility in the adult life. In the following paragraph, we will briefly review the genetic networks regulating the differentiation of DA and 5-HT neurons during embryonic brain development. We will then summarize our recent findings supporting the idea that an altered maturation of these two neurotransmitter pathways can markedly affect seizure susceptibility in the adult brain.

DEVELOPMENT OF DOPAMINERGIC AND SEROTONERGIC NEURONS

In the mammalian nervous system individual population of neurons develop in a stereotypic position identified by their coordinates along the antero-posterior and dorso-ventral axes [22, 23]. Three organizing centers, the mid-hindbrain boundary (MHB or isthmus), the floorplate (FP), and the anterior neural ridge (ANR) control regionalization of the two main axes and specify the location and the cell fate of specific neuronal population within the brain fate map [24]. This is also true for dopaminergic (DA) and serotonergic (5-HT) neurons localized in caudal midbrain and rostral hindbrain, respectively [22].

Regionalization of midbrain/hindbrain territory. The MHB is anatomically characterized as a constriction between midbrain and hindbrain, although its precise anatomical boundaries have not yet been determined. For this reason the borders of the MHB are only defined with the use of expression patterns of specific genes, which delineate its competence territory (Figure 1). By embryonic stage 7.5 (E7.5) in mouse, the transcription factors Otx2 and Gbx2 are expressed in a complementary fashion in the embryo: the border along their expression territories delineates the future junction between mesencephalon and metencephalon: the MHB (Figure 1). At early stages Fgf8, Wnt1 and Otx2 are expressed in the caudal midbrain regions that give rise to midbrain

DA neurons. In contrast, Fgf8 and Gbx2, but not Wnt1, are expressed in the region that gives rise to rostral 5-HT progenitors (Figure 1). The transcription factors Engrailed1 (En1) and Engrailed2 (En2) are instead expressed in both caudal midbrain and anterior hindbrain. The expression domain of each gene reflects the role the gene plays in the formation of this territory. Otx2 and Gbx2 expression domains are restricted to the anterior and posterior part of the neural tube and, by doing so, define positioning of MHB along the anterior-posterior axis; Wnt1 and Fgf8 expression patterns are restricted to the mid-hindbrain junction in specular domains and are involved in the growth and maintenance of MHB cells; genes expressed across the entire mid- and hindbrain territory, such as Pax2, Pax5, En1/2, define the identity of this region, as a whole [25-27]. The second organizing center of the midbrain/hindbrain region is the FP. Sonic hedgehog (Shh), the key-signaling molecule of the FP, is mainly supplied to the neural tube by the ventral midline structures. During neurogenesis, dopaminergic and serotonergic neuron progenitors within the neuroepithelium are committed by the combined action of Fgf8 and Shh, originating form the MHB and the FP, respectively.

Determination and differentiation of dopaminergic and serotonergic progenitors. The concomitant action of MHB and FP activates in the midbrain a combination of transcription factors including Otx2, Lmx1a/b, En1/2, Msx1/2, Ngn2 and Mash1, in a temporal sequence. The expression of Otx2, Lmx1b and En1/2 genes is already initiated by E9.0 [28-31]. Subsequently, Lmx1a and Msx1/2 expression turns on around E9.5, while Ngn2 and Mash1 are not expressed until E10.75 [32]. While Otx2 and En1/2 participate to the positioning of the MHB region, recent studies identified Lmx1a and Msx1 as determinants of midbrain DA neurons [33]. The two transcription factors are induced by Shh, either directly or through an unknown signal, in ventral midline cells in the mesencephalon. However at least in the intermediate and posterior ventral midbrain activation of Lmx1a and Msx1/2 appears to depend on the presence of Otx2 [34], suggesting that Otx2 might be required for direct activation of Lmx1a and Msx1/2 and/or to provide DA progenitors with competence in responding to the Shh by inducing the expression of Lmx1a and Msx1/2. Furthermore, while Lmx1a is sufficient to induce DA cell differentiation in ventral progenitors cells and induces the expression of Msx1/2, Msx1/2 seem to be instead involved in the repression on the lateral progenitors cell fate. Floor plate (Shh-positive) cells can turn into dopaminergic progenitors through the acquisition of neuronal potential and down-regulation of Shh, a step that involves the activation of the proneural genes Ngn2 and Mash1 by the combined action of Lmx1a and Msx1/2 [33, 35].

Recent reports have suggested that mesencephalic floor plate cells have indeed the potential to directly generate mesencephalic dopaminergic neurons whose A-P identity depend on the expression of Otx2 [36]. Despite several reports demonstrate an early requirement for Shh, supporting DA progenitors through the induction of Lmx1a, recent evidence indicate an additional, later role of Shh to inhibit midline (DA) progenitors proliferation and neurogenesis [37]. In this context Wnt1 might have a relevant role in regulating cell proliferation. This is supported by the finding that mouse mutants lacking or over-expressing Otx2 respectively exhibit loss or increase in both Wnt1 expression and proliferating activity [34, 38]. Midbrain DA neurons would be therefore

specified dorso-ventrally as FP cells and antero-posteriorly by Otx2 signals while hindbrain neurons, such a 5-HT cells, would originate from precursors lacking the Otx2 signal [36, 39].

A number of genes important for final differentiation and long-term maintenance of DA neurons start to be expressed in immature DA neurons including the homeobox genes Lmx1b, Pitx3 and En1/2 and the nuclear orphan hormone receptor Nurr1. Loss of function of these genes results in the loss of dopaminergic neurons after birth [30, 40, 41]. During maturation of DA neurons, other genes necessary for the synthesis and homeostasis of DA are expressed including, tyrosine hydroxylase (Th), aromatic amino-acid decarboxylase (Aadc), vescicular monoamine transporter 2 (Vmat2), dopamine D2 receptor (D2r) and dopamine transporter (Dat) as well as other genes including c-ret and glial cell-line derived neurotrophic factor receptor α-1 (Gdnfrα-1), the receptor complex for the neurotrophic factor glial cell line-derived neurotrophic factor (Gdnf).

Rostral hindbrain 5-HT neurons, like midbrain DA neurons, have been shown to depend on both the activity of the MHB and the FP. However a third signal, Fgf4, coming from the primitive streak, participate to the specification of hindbrain 5-HT progenitors. Expression of Nkx2.2 is then essential for specification of 5-HT neurons and ventral progenitor identity, conferring competence to become 5-HT neurons [42, 43]. Nkx2.2 is a homeodomain transcription factor expressed in the ventral-most neuroepithelium in response to Shh signaling. Once the position of the precursors is defined, other transcription factors are required to establish the serotonergic phenotype. These transcription factors are expressed in postmitotic cells, and comprise a Lim homeodomain gene Lmx1b, and a transcription factor Pet1. Pet1 has a unique expression pattern: it is strictly limited to the raphe nuclei, and appears one day before the serotonergic neurons can be identified. This factor could directly activate the transcription of the genes that define the 5-HT phenotype: tryptophan hydroxylase (Tph), aromatic amino acid decarboxylase (Aadc), the 5-HT transporter (Sert) and the vesicular monoamine transporter (Vmat) [43].

The MHB organizer not only determines the competence of the territory to develop certain neuronal populations (dopaminergic and serotonergic), but also defines the compartments where progenitor cells are to be positioned along the anterior-posterior and dorso-ventral axes. Changing position and extension of the MHB territory, by shifting Otx2 or Gbx2 expression domains, can either expand or reduce the DA or 5-HT neuron population [39, 44-46]. Furthermore, Otx2 has been shown to be required for midbrain DA neuron generation independently of controlling isthmic organizer positioning, suggesting that Otx2 may determine the A-P identity of neural progenitors that confer DA neuron identity [39, 47]. In particular, manipulations of Otx2 expression domain result in anterior to posterior ($En1^{Cre/+}$; $Otx2^{flox/flox}$ mice; [39]) or dorsal to ventral ($Otx1^{Cre/+}$; $Otx2^{flox/flox}$ mice; [46]) transformation of the cell fate with the consequent alteration of positioning and extension of DA and 5-HT neuronal population. Figure 1 summarizes the genetic networks controlling the differentiation of DA and 5-HT neurons during embryonic brain development.

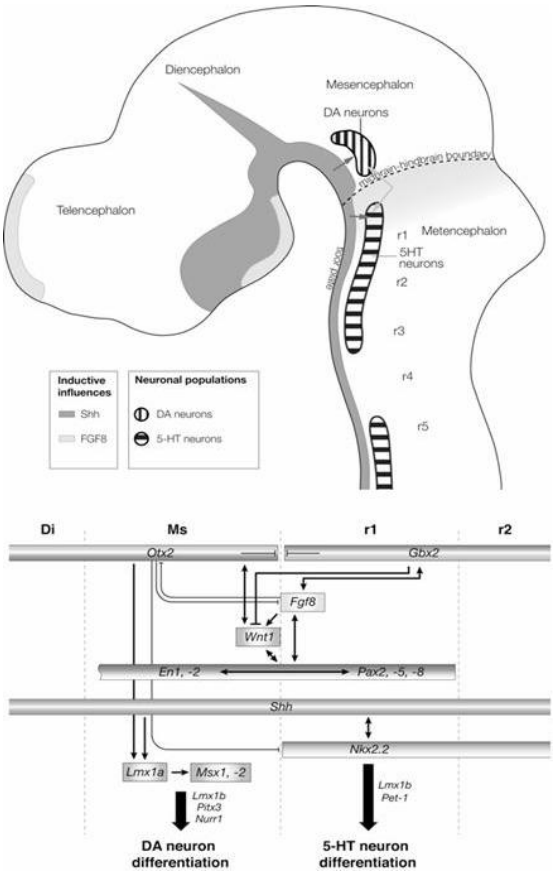

Figure 1. Gene regulatory networks involved in DA and 5-HT neuron differentiation.
(Top) Patterning signals in the developing brain. Sagittal view of an E11 mouse embryo neural tube; anterior is on the left. Expression of the secreted factors fibroblast growth factor 8 (Fgf8), Wnt1 and sonic hedgehog (Shh) is depicted at the MHB, in the anterior neural ridge and ventral diencephalon and within the floor/basal plate of the spinal cord, hindbrain, midbrain and caudal forebrain. Mesencephalic dopaminergic (DA) neurons are induced by a combination of Fgf8 and Shh (arrows). 5-HT neurons are specified by a combination of the same factors but they require an early inductive signal (Fgf4, not shown) derived from the anterior mesoderm during gastrulation.
(Bottom) Gene expression patterns participating to DA and 5-HT neuron differentiation; anterior is on the left. Gbx2 expression maintains Fgf8 expression, whereas Otx2 and Gbx2/Fgf8 regulate each other negatively. Concomitantly, the expression territories of Fgf8, Wnt1, Engrailed and Pax genes become interdependent and establish a positive regulatory loop that is necessary to maintain MHB identity. The mid-diencephalic border is positioned by negative cross-regulations of Engrailed/Pax and Pax6 (not shown), whereas Fgf8 exerts a negative influence on the caudal expression of Hox genes (not shown). Later on, Shh induces the expression of Lmx1a and Msx1/2. While Lmx1a is sufficient to induce DA cell differentiation of ventral progenitors cells and induces the expression of Msx1/2, Msx1/2 is involved in the repression on the lateral progenitors cell fate (not shown). Midbrain DA neurons are specified dorso-ventrally by Shh signaling and antero-posteriorly by Otx2 signals, while 5-HT cells originate from precursors lacking the Otx2 signal. Shh signaling induces the expression of Nkx2.2, which is then essential for specification of 5-HT neurons and ventral progenitor identity, conferring competence to become 5-HT neurons. Once positioning and identity of the neuronal precursors are determined, specific differentiation programs are activated in DA (Lmx1b, Pitx3, Nurr1) and 5-HT neurons (Lmx1b and Pet-1). Adapted from [50, 51]. See text for details. Abbreviations: Di, diencephalons; Ms, mesencephalon; r, rhombomeres.

ALTERED DEVELOPMENT OF DA AND 5-HT NEURONS CAN AFFECT SEIZURE SUSCEPTIBILITY: INDICATIONS FROM MUTANT MICE

We recently investigated seizure susceptibility in mutant mice with conditional inactivation of the Otx2 gene in DA precursor cells. In these mice, Otx2 was conditionally inactivated by a Cre recombinase expressed under the transcriptional control of the Engrailed1 (En1) gene ($En1^{Cre/+}$; $Otx2^{flox/flox}$), resulting in a reduced number of DA neurons and an increased number of 5-HT neurons in the ventral midbrain that persists until adult age [39, 48]. In particular, adult $En1^{Cre/+}$; $Otx2^{flox/flox}$ mice showed a massive increase of 5-HT in the pons, ventral midbrain, hippocampus (CA3 subfield) and cerebral cortex, that was paralleled by reduced levels of 5-HT transporter (Sert) in the same areas. Due to this increased 5-HT hyper-innervation, $En1^{Cre/+}$; $Otx2^{flox/flox}$ mice were resistant to generalized seizures induced by the glutamate agonist kainic-acid (KA) (Figure 2). Brain 5-HT depletion in mutant mice restored 5-HT content to control levels, fully re-establishing KA-seizure susceptibility [48].

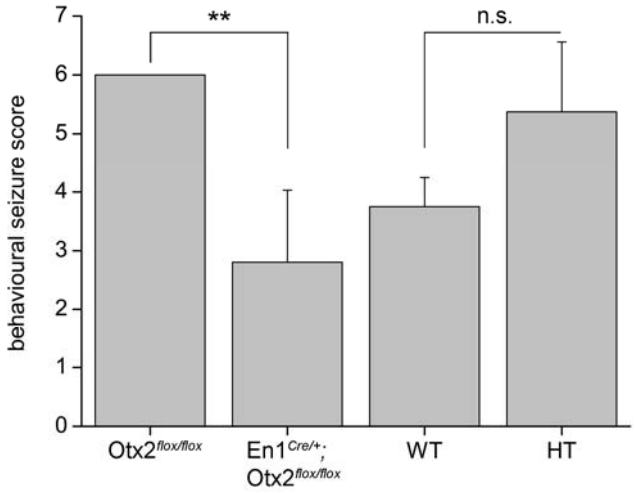

Figure 2. Seizure susceptibility in mutant mice with altered development of DA and 5-HT neurons. $En1^{Cre/+}$;$Otx2^{flox/flox}$ mice show a marked resistance to kainic acid (KA)-induced seizures, as compared to their controls ($Otx2^{flox/flox}$ mice). Conversely, no significant difference in KA seizure score is detectable between $En1^{+/-}$; $En2^{-/-}$ (HT) mice and their wild-type (WT) controls (C57Bl/6x129Sv mixed genetic background). $Otx2^{flox/flox}$ control mice have a higher KA susceptibility respect to WT mice since they are generated in the KA-sensitive DBA2 background [48]. Bars represent the maximum seizure rating scale value scored by each genotype (n = 8-10 animals per group) over a period of two hours after intraperitoneal (i.p.) administration of KA (20 mg/kg). Data are expressed as mean ± s.d. ** $p<0.001$, t-test; n.s., not statistically significant difference ($p>0.05$, t-test). Seizures were scored as described in [48]: 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. Data for $Otx2^{flox/flox}$ and $En1^{Cre/+}$;$Otx2^{flox/flox}$ mice are re-adapted from [48].

In parallel experiments, we also evaluated KA seizure susceptibility in $En1^{+/-}$; $En2^{-/-}$ mutant mice (HT mice), which display a post-natal and progressive loss of DA neurons of the substantia nigra [49]. It is important to point out that the post-natal DA cell loss in HT mice is not accompanied by increased number of 5-HT cells (our unpublished observations). HT mice did not show significantly altered seizure susceptibility when compared to control (Figure 2).

CONCLUSIONS

Altered expression and function of homeobox genes during brain development may lead to abnormal specification of brain areas, neuronal identity, circuit formation, ultimately leading to an imbalance between excitation and inhibition. Our studies, carried out in mutant mouse lines lacking Otx2 or Engrailed1/2 genes, show that altered specification of DA and 5-HT cell fate results in altered seizure susceptibility in the adult age. Classical pharmacological studies indicate that both DA and 5-HT may have an anti-epileptic action. It might be therefore questioned that reduction of DA cells in both $En1^{Cre/+}$; $Otx2^{flox/flox}$ and HT mice could contribute to lower seizure susceptibility in these animals. On the contrary, $En1^{Cre/+}$; $Otx2^{flox/flox}$ mice were markedly resistant to KA seizures due to 5-HT hyper-innervation, whereas HT mice (in which 5-HT levels were unchanged) showed a normal susceptibility to KA seizures. These results suggest that the effect of 5-HT hyper-innervation onto seizure control is more prominent than that of DA reduction.

ABBREVIATIONS

ANR, anterior neural ridge;
Aadc, aromatic amino-acid decarboxylase;
CNS, Central Nervous System;
DA, dopamine;
Dat, dopamine transporter;
D2r, dopamine D2 receptor;
E, embryonic stage;
En, Engrailed;
Fgf, fibroblast growth factor;
FP, foorplate
Gdnf, glial cell line-derived neurotrophic factor;
Gdnfrα-1, glial cell-line derived neurotrophic factor receptor α-1;
HT mice, $En1^{+/-}$; $En2^{-/-}$ mutant mice;
5-HT, 5-hydroxytryptamine (serotonin);
8-OH-DPAT, 8-hydroxy-2-(di-n-propylamino) tetralin;
KA, kainic acid;
MHB, mid-hindbrain boundary;

PCA, para-chloroamphetamine;
pCPA, para-chlorophenilalanine;
Sert, serotonin transporter;
Shh, Sonic hedgehog;
Th, tyrosine hydroxylase;
Tph, tryptophan hydroxylase:
Vmat, vescicular monoamine transporter;

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