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Spontaneous Epileptic Manifestations in a DCX Knockdown Model of Human Double Cortex

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Previous reports indicate that in utero knockdown of doublecortin (DCX) results in the genesis of a subcortical heterotopia reminiscent of the doublecortex observed in female patients with DCX mutations. It has also been shown that these rats display an increased susceptibility to convulsant agents and increased cortical neurons excitability; but it is presently unknown whether they display spontaneous seizures. Furthermore, the link between the size of heterotopia and the clinical manifestation remained to be elucidated. Using video–electrocorticogram recordings, we now report that DCX knockdown induces frequent spontaneous seizures commonly associated with myoclonic jerks in adult rats. Surprisingly, epilepsy occurred even in rats with very small subcortical heterotopias, as revealed by histological analysis of recorded animals. Moreover, the severity of the epileptic manifestations was positively correlated with both the size of the subcortical heterotopia and the age of recorded animals; thus, epileptic features were not detected in immature affected rats. In conclusion, our data demonstrate for the first time that subtle alterations can yield epilepsy and reveal a strong correlation between thicknesses of subcortical heterotopia, age of affected individuals and clinical impairment.

Keywords: cortical dysplasia, doublecortex, in vivo telemetry, migration disorder, partial epilepsy

Introduction

Abnormal cortical development is increasingly recognized as a cause of developmental disabilities and epilepsy (Barkovich et al. 2005). The pathophysiological mechanisms relating cortical malformations to epilepsy remain elusive. This is essentially due to the lack of reliable experimental models of cortical malformations and the limited access to human brain tissue for fine physiological analysis and investigation.

Mutations of doublecortin (DCX), a microtubule-stabilizing protein expressed in immature neurons that is important for migration (Francis et al. 1999; Bai et al. 2003; Tanaka et al. 2004), are among the best characterized genetic disruptions leading to lissencephaly and subcortical band heterotopia in humans (Reiner et al. 1993; des Portes et al. 1998; Gleeson et al. 1998; Pilz et al. 1999). Although functional DCX knockout models have been generated, progress has been hindered by the fact that these animals displayed little or no dysgenesis in the neocortex (Corbo et al. 2002; Kerjan et al. 2009). These knockout models display in contrast hippocampal dysplasias (Hirotsune et al. 1998; Fleck et al. 2000; Nosten-Bertrand et al. 2008; Kerjan et al. 2009) and allowed physiological investigations on the contribution of hippocampal alterations to brain function and pathology (Nosten-Bertrand et al. 2008; Kerjan et al. 2009). However, whether human patients display similar hippocampal dysplasias is presently uncertain (see Kappeler et al. 2007). Similar comments apply to the analysis of knockout (KO) mice for LIS-1 (Fleck et al. 2000; Jones and Baraban 2007), another cytoskeleton protein involved in lissencephaly.

A rat model of band heterotopia has been developed by knocking down *DCX* expression in nascent layer 2/3 neurons by using in utero ribonucleic acid (RNA) interference (Bai et al. 2003). Neurons transfected with short hairpin RNAs (shRNAs) targeting *DCX* form ectopic clusters in the white matter and deep cortical layers similar to that observed in female patients with DCX mutations, thus providing a valuable animal model of this condition. In addition, DCX knockdown rats display a clear increased cortical excitability (Ackman et al. 2009) and susceptibility to develop generalized seizures as depicted by the test of subconvulsive repeated doses of the convulsant agent pentylenetetrazol (Manent et al. 2009). These data suggest that cortical networks in DCX knockdown rats are pro-epileptogenic. However, it is presently unknown whether these animals are spontaneously epileptic and display seizure events with similar electroclinical manifestations to that described in human patients with subcortical band heterotopia. To investigate this issue, we performed video behavioral analyses and telemetric electrocorticography (ECoG) recordings from freely moving adult DCX knockdown rats, which were subsequently investigated for the presence of histological alterations in the cortex. We show that animals with subcortical heterotopia display frequent spontaneous seizures thus providing a unique opportunity to investigate the pathophysiological basis of epilepsy after DCX mutations. In this animal model, we observed epileptic manifestations even in rats with very small heterotopias, in agreement with a previous case report (de Portes et al. 1998), and a strong correlation between heterotopia thickness and epileptic features. We show further that the severity of the epileptic manifestations worsens with age. In conclusion, our data demonstrate for the first time that subtle alterations can yield epilepsy and also reveal that the severity of epilepsy correlates with the severity of cortical alterations and aggravate with age in this rat model of subcortical band heterotopia.

Materials and Methods

In Utero Electroporation

Wistar rats (Janvier, Le Genest-Saint-Isle, France) were mated, cared, and used in our animal facilities in agreement with the European Union and French legislations. Timed pregnant rats (embryonic day 15 [E15])

were anesthetized with a mixture ketamine/xylazine (respectively at 100 and 10 mg/kg). The uterine horns were exposed, and a lateral ventricle of each embryo injected via pulled glass capillaries and a microinjector (Picospritzer II; General Valve Corporation, Fairfield, NJ) with Fast Green (2 mg/mL; Sigma, St Louis, MO) combined with the following DNA constructs: 1.5 mg/mL pCAGGS–green fluorescent protein (GFP) alone or combined with 1.5 mg/mL of shRNA targeting the 3′-untranslated region of *DCX* described before (Bai et al. 2003). This step was followed by in utero electroporation by discharging a 500-F capacitor charged to 70 V with a BTX exponential decay wave electroporator (BTX Harvard Apparatus, BTX Molecular Delivery Systems, Holliston, MA). The voltage was discharged across copper alloy oval plates placed on the uterine wall across the head of the embryo. Injections and electroporations were all done in the same side.

Explorative Behavior

All experiments were conducted in accordance with the national and European (86/609/EEC) laws for the use of animals in research and were approved by the local ethical committee (Landesuntersuchungsamt Koblenz, 23 177-07/G07-1-001). Thirteen DCX knockdown (6 males and 7 females, between 250 and 4 wild-type (WT) Wistar rats (400–500 g) were used for this study. In order to test the locomotion and exploration activity of the animals, they were placed 10 min for 5 consecutive days (between 13:00 and 15:00 h) in an open field (60 × 70 cm^2), and a behavioral recording was made with EthoVision (Noldus Information Technology, Berlin, Germany). The first 2 sessions were performed in an empty arena whereas the next 3 ones were done in the presence of 2 unknown objects. Two sessions were recorded with 2 similar objects, and the last day one was exchanged by a novel object. *P* values were calculated with parametric unpaired samples *t*-tests (Systat Version 10, Systat Software, Erkrath, Germany) after testing the Gaussian distribution.

Video–ECoG Recordings of Adult Rats

Adult animals of ages ranging from 209 to 354 days old were implanted with a telemetric recording system as described previously (Lapray et al. 2008). Briefly, after being deeply anesthetized with a mixture of chloralhydrate (Sigma-Aldrich, Steinheim, Germany) and ketamine (500 mg/mL; ratiopharm GmbH, Ulm, Germany), a transmitter was placed in the abdominal cavity and wires were slipped between muscles and skin up to the head, where another incision was made to expose the skull. The tips of the wires were used as electrodes and placed directly on the dura. The recording electrode was placed (in contact with the dura) above the barrel cortex of the electroporated hemisphere (lateral [L] = bregma + 5.5 mm, anteroposterior [AP] = bregma – 2.3 mm) (Paxinos and Watson 1998). The reference and the ground electrodes were placed (in contact with the dura) above the cerebellum (L = bregma + 2 mm, AP = bregma – 11.5 mm and L = bregma – 1.5 mm, AP = bregma + 11.5 mm, respectively). This assembling was fixed with blue light sensitive cement (Gluma Comfort Bond, Heraeus Kulzer GmbH, Hanau, Germany and Ivoclar Vivadent AG, Schaan, Liechtenstein). Both incision sites were closed using 4-0 Resolon (Resorba, Nürnberg, Germany). Surgery lasted a maximum of 3 h from induction of anesthesia. After surgery, a recovery period of 1 week was given to the animals before starting the first recording session, corresponding to the time needed to gain the presurgical weight. Behavioral studies were performed as described previously (Lapray et al. 2009). The animals were then placed 1 h per day in an empty cage (37 × 20 × 30 cm) between 13:00 and 16:00 h for 3 consecutive days. The animals were also recorded for one session of 1 h in an enriched environment to test whether novelty could enhance spontaneous seizures. In order to control that the implantation of the recording system as well as the experimental procedure did not induce any spontaneous seizures, 2 WT Wistar rats were implanted and followed the same experimental protocol as the DCX knockdown animals. ECoG signals (recorded continuously at a sampling rate of 1000 Hz) and video data (25 frames/s, 720 × 576 pixels) were collected simultaneously and stored on a personal computer via a Cambridge Electronic Design and Spike2 software (Cambridge Electronic Design, Cambridge, UK). The data were then imported to MatLab (MatLab 7, The MathWorks Inc., Natick, MA) for further analysis.

ECoG Recordings of Rat Pups

Ten-day-old pups (n = 17) were implanted with cortical and hippocampal electrodes under urethane anesthesia (2 g/kg, intraperitoneally [i.p.]) and local anesthesia (0.5 mL of 1% novocaine, subcutaneously). For recording of cortical and hippocampal ECoGs, insulated 80 μm nichrome microwires were imbedded into the S1FB (AP = 1.2, L = 3.5, horizontal [H] = 1.0) and CA1 field (AP = 2.6, L = 1.2, H = 3.0). The reference electrode was screwed into the occipital bone. After the surgery, the animals were kept on artificial nutrition for 1 day and after that they were returned to their mothers.

ECoG recordings started at P12–14 on freely moving animals; they were amplified with a World Precision Instruments Inc. (Berlin, Germany) amplifier and recorded at a sampling rate of 10 KHz with Clampex 9.0 software (PClamp, Axon Instruments, MDS Analytical Technologies Limited, Wokingham, UK). After recording of the ongoing spontaneous activity, injection of pentylenetetrazole (PTZ; Sigma), a γ-aminobutyric acid A receptor antagonist, was used to evaluate the susceptibility to seizures in control and DCX pups. The animals were injected twice with a total dose of 50 mg/kg (each injection consisted of 25 mg/kg PTZ, i.p.; period between injection 10 min) (Weller and Mostofsky 1995; Manent et al. 2009). Latency, duration, and intensity of seizures were measured.

Histology

Brains from all analyzed rats were fixed with paraformaldehyde (4% in phosphate-buffered saline [PBS]). Brain sections (50 μm) were obtained with a vibratome (Leica Microsystems GmbH, Wetzlar, Germany) and permeabilized for 10 min at room temperature (RT) in PBS–Triton X-100 (0.1%)–goat serum (5%). After permeabilization, slices were incubated overnight at RT with rabbit anti-NeuN (1:1000; Chemicon, Millipore, Billerica, MA) and mouse anti-GFP (1:2000; Molecular Probes, Eugene, OR). The slices were rinsed 3 times in PBS and incubated for 2 h at RT in Cy-3–conjugated goat anti-rabbit antibodies and donkey anti-mouse conjugated with fluorescein isothiocyanate (1:200; Jackson ImmunoResearch, West Grove, PA) and mounted. Sections were examined under a Olympus fluoview 500 laser-scanning microscope (Olympus Optical, Tokyo, Japan) using ×10, ×20, and ×65 objectives; images were digitized using the built-in software and exported in tiff format for quantitative analysis with the analysis software ImageJ 1.33d (W. Rasband, National Institutes of Health).

Results

Adult DCX Knockdown Rats Are Hyperactive

In order to study the behavior and overall activity of adult DCX knockdown rats displaying heterotopias (n = 13 see below) in comparison to age-matched controls (n = 4), single rats were placed in an empty open field for 10 min. The overall activity of the 13 DCX knockdown animals was significantly higher than the overall activity in the controls (Fig. 1). The DCX

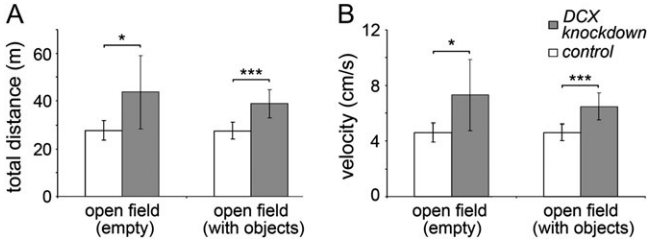

Figure 1. Differences in the behavior between control and DCX knockdown adult rats in the open field. Bar diagrams illustrate the average total running distance during an observation period of 10 min (*A*) and the average running velocity (*B*) in the empty open field for 4 control (open bars) and 13 DCX knockdown rats (filled bars). The open field was known to the animal and did not contain any objects (left) or contained 2 unknown objects (right). Data are expressed as mean ± standard deviation. Statistically significant differences at the $P < 0.05$ level are marked with * and at $P < 0.001$ level by *** (*t*-test).

Table 1
Summary of the ECoG recordings in the cage and the behavior in the empty open field for the 13 DCX knockdown rats

Animal #	Gender	Seizures per hour	Average seizure duration (s)	Max amplitude (μV)	Normalized max amplitude	Average amplitude (μV)	Normalized average amplitude	Max frequency (Hz)	Total distance (cm)	Mean velocity (cm/s)
1	F	43.00	11.46 ± 7.96	658.68 ± 69.02	2.06 ± 0.2	78.59 ± 11.22	2.44 ± 0.4	8.64 ± 1.61	3892.07	6.55
2	F	37.33	10.69 ± 8.56	484.12 ± 69.13	2.22 ± 0.4	45.69 ± 6.97	2.24 ± 0.37	13.97 ± 6.82	4641.72	7.92
4	F	23.67	10.17 ± 7.12	735.48 ± 45.76	1.71 ± 0.16	108.59 ± 17.02	2.62 ± 0.42	8.22 ± 1.18	4697.29	7.83
7	M	25.00	26.63 ± 18.72	754.97 ± 45.58	2.19 ± 0.15	109.70 ± 11.59	3.09 ± 0.32	8.55 ± 1.69	4292.35	7.16
8	M	15.67	13.92 ± 4.95	663.65 ± 24.90	3.09 ± 0.43	72.56 ± 7.53	3.46 ± 0.47	8.89 ± 2.71	4191.68	6.99
9	M	25.33	6.65 ± 4.45	366.46 ± 59.44	2.53 ± 0.57	33.70 ± 3.57	2.29 ± 0.25	14.22 ± 3.99	3741.01	6.24
10	F	34.67	9.19 ± 5.12	726.30 ± 39.40	2.20 ± 0.32	94.11 ± 7.38	2.67 ± 0.24	13.85 ± 4.91	3018.50	5.03
12	F	24.00	16.51 ± 15.28	701.73 ± 62.86	1.52 ± 0.36	89.43 ± 13.57	2.26 ± 0.41	8.45 ± 1.61	6143.81	10.24
13	F	19.00	8.99 ± 8.85	635.90 ± 89.74	2.45 ± 0.45	74.27 ± 10.57	2.97 ± 0.45	10.73 ± 3.89	3593.32	5.99
18	F	22.33	4.80 ± 2.06	455.94 ± 60.72	1.15 ± 0.16	47.50 ± 7.00	1.19 ± 0.12	18.55 ± 6.58	3159.41	2.27
19	M	25.33	4.30 ± 2.20	583.62 ± 73.55	1.51 ± 0.20	74.95 ± 12.38	2.14 ± 0.35	8.61 ± 1.35	3811.32	6.36
20	M	9.67	4.46 ± 2.64	424.38 ± 80.77	2.32 ± 0.50	41.26 ± 7.78	2.23 ± 0.43	10.04 ± 2.43	5034.32	8.39
21	M	14.33	6.50 ± 2.65	505.34 ± 69.91	1.42 ± 0.18	51.89 ± 5.91	1.84 ± 0.18	11.52 ± 5.78	4120.19	6.87

NOTE.—Each value is expressed as mean ± standard deviation. As behavioral parameters, the total running distance (cm) and the average velocity (cm/s) measured in the empty open field are listed. The open field experiments were done before the surgery that is with nonimplanted DCX knockdown rats. "Normalized maximal seizure amplitude" was calculated from maximal seizure amplitude divided by the maximal wakefulness amplitude, and "normalized average seizure amplitude" was calculated from average seizure amplitude divided by average wakefulness amplitude. M = male and F = female.

knockdown animals ran on average a total distance of 4379 ± 1534 cm with an average velocity of 7.32 ± 2.56 cm/s whereas the controls moved 2771 ± 401 cm with a velocity of 4.62 ± 0.67 cm/s (P = 0.0023 and 0.0022, respectively). During the recording sessions with new objects in the arena, the DCX knockdown group was also significantly more active than the control group (P < 0.0001 for both distance and velocity). This effect was not directly correlated with novelty because the 2 groups did not display any significant differences in the time spent exploring objects.

Adult DCX Knockdown Rats Display Spontaneous Seizures

Sham-treated (i.e., rats electroporated with GFP; n = 10) and control adult rats (n = 2) never displayed epileptic manifestations or alterations in the ECoG. In contrast, all electroporated DCX knockdown animals tested in adulthood and presenting heterotopias (n = 13; see below) displayed frequent spontaneous seizures. Seizures were defined as events of high amplitude (larger than 3× the standard deviation calculated from the ratio seizure amplitude/wakefulness amplitude) and of more than 2 s duration. These epileptic activities were associated with myoclonic jerks of the jaw in some cases (see Supplementary Movie) and with tail and back clonus in one animal (Table 1). These animals displayed on average 24.6 ± 9.4 spontaneous ECoG epileptic events per hour (median of 3 recording periods of 1 h duration on 3 consecutive days) with an average duration of 10.4 ± 5.9 s (see example in Fig. 2). The analysis of concomitant motor manifestations, mainly myoclonic movement of the jaws, revealed the presence of spontaneous frequent motor seizures (2.95 ± 1.09 motor seizures/h of 12 ± 1.9 s duration). Therefore, about 88% of the recorded events were subclinical.

In order to test the influence of novelty on the expression of spontaneous seizures, DCX knockdown animals were recorded for 1 h in an enriched environment. This protocol did not affect the number of seizures but the latency before the first event when compared with the cage recordings (latency in the cage 272 ± 67 s and in the open field 1057 ± 217s, P = 0.0002). The first seizure always appeared in both recording conditions during quiet wakefulness that appeared after a longer period in the novel environment.

Adult DCX Knockdown Rats Display Subcortical Heterotopias

At the end of the recording sessions, animals were processed for histological analyses in order to detect ectopic neurons (Fig. 3). At the time when histological analysis was performed, animals were more than 6 months old and transfected neurons had lost GFP expression. Because GFP antibodies could no longer detect the presence of GFP, analysis was performed on brain sections stained with NeuN antibodies, a neuron-specific nuclear marker. This allowed us to visualize groups of ectopic neurons in the white matter (Fig. 3) but not to detect ectopic neurons in deep cortical layers (electroporated neurons were initially fated to upper cortical layers; see Ackman et al. 2009), so that the size of the heterotopic masses was rather underevaluated (also see Fig. 4). Only DCX knockdown animals presenting subcortical heterotopias were thus considered for analysis. In this group of animals, the size of the heterotopia ranged from 0.12 to 128.76 mm^3 (mean value was 12.68 ± 9.69 mm^3). In the majority of the cases (11 out of 13), subcortical heterotopias were localized between –1.30 and –5.20 mm with respect to the bregma as defined by the rat atlas of Paxinos and Watson (1998) and centered directly under the parietal cortex (somatosensory cortex). The 2 remaining cases (cases #2 and 7) presented ectopias that extended from +1.20 to –5.20 and, in addition to the white matter of the somatosensory cortex, ectopia also invaded the white mater under the motor cortex (Fig. 3*C*). Age-matched sham-treated animals (n = 10 rats electroporated with GFP) never displayed ectopic masses of neurones in the white mater or any signs of cortical disruption.

Correlation Analysis between Seizure Events versus Heterotopia Degree

As mentioned above, adult DCX knockdown animals displayed variable degrees of epileptic manifestations and heteropia volume. We then investigated whether these parameters were correlated. A positive correlation was found between the volume of the heterotopia and the mean duration of epileptiform events (Pearson correlation coefficient = 0.806; P = 0.0008); no significant correlations were found between heterotopia volume and frequency or amplitude of epileptiform events. We also compared ECoG values versus degree of

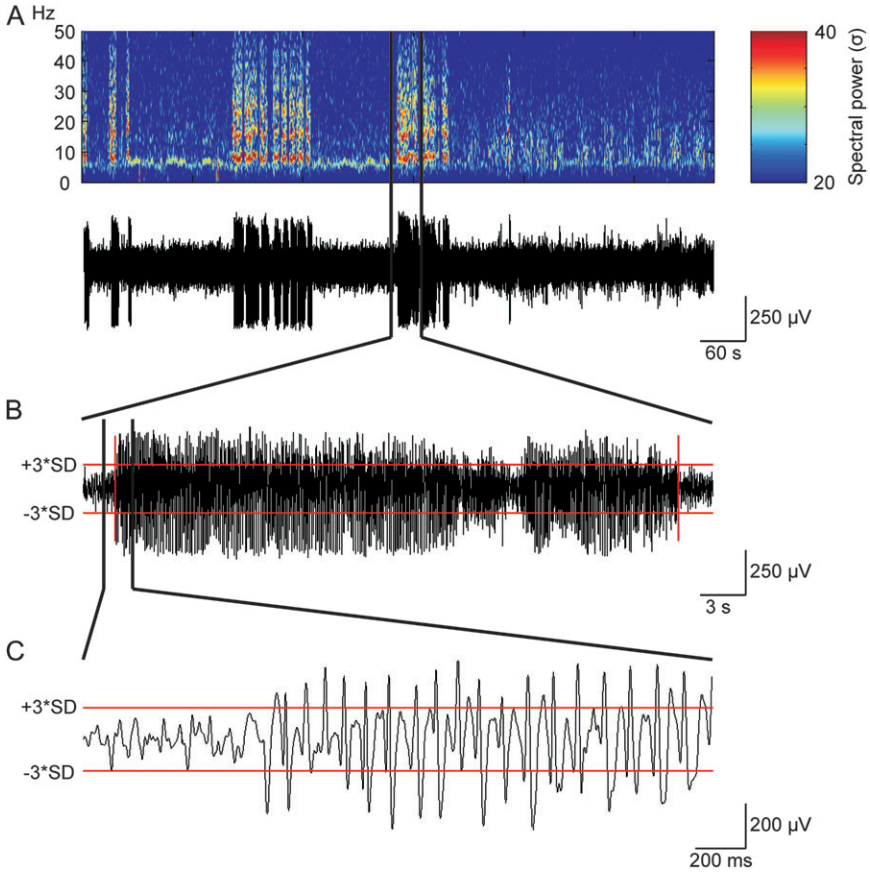

Figure 2. Seizure activity recorded in an adult DCX knockdown rat. (*A*) ECoG recording (filtered between 1 and 50 Hz) and corresponding spectrogram analysis. Seizures appeared only during the first half of the recording when the animal was awake (theta range activity, 6–8 Hz). No seizure event was detected during the second half of the recording when the animal displayed slow wave sleep activity (characterized by spindle bursts in the beta range and the disappearance of theta). (*B*) One epileptic event with duration of ~38 s and a maximum amplitude of 765 µV from trace shown in (*A*) at higher temporal resolution. Activity exceeding 3-fold the standard deviation was considered to be part of the seizure. (*C*) The first second of the epileptic discharge shown in (*A* and *B*).

cortical alteration by segregating animals within 3 groups according to the volume of heterotopia: small (volume inferior to 1 mm^3; $n = 6$, Fig. 3*A*), medium (volume ranging from 1 to 10 mm^3; $n = 6$, Fig. 3*B*), and large heterotopia (volume greater than 10 mm^3; Fig. 3*C*). Again a positive correlation was found between the degree of cortical alteration and the mean duration of events (Pearson correlation coefficient = 0.722; $P = 0.003$, Fig. 3*F*) and their amplitude (Pearson correlation coefficient = 0.641; $P = 0.013$, Fig. 3*E*).

Developing Rats Did Not Display Spontaneous Seizures but Exhibited an Increased Susceptibility to PTZ

The previous data depicted a strong link between subcortical heterotopia and epilepsy in DCX knockdown adult rats. Because the age of seizure onset in patients with band heterotopia ranged from neonates to teenagers (Barkovich et al. 1994; Tanaka and Gleeson 2007), we investigated whether epileptic manifestations are already present in "infantile" rats by analyzing DCX knockdown rats at the end of the second postnatal week. Because their size was not compatible with the telemetric recording system used for adults, these pups were recorded using conventional ECoG protocols (see Methods). From 17 DCX knockdown pups recorded at P12–15, none displayed spontaneous epileptic manifestations. Post hoc analysis revealed the presence of heterotopias in only 4 pups (around 30% of injected embryos developed white matter heterotopia in our hands); the average size of heterotopia in this group being below 1 mm^3. At this age, GFP expression could still be detected (Fig. 4) allowing the identification of transfected neurons. They localized in the white matter and within heterotopic masses as described before (Ackman et al. 2009) and are also found ectopically in cortical layer VI (transfected neurons at E15 are committed to upper cortical layers). These data strongly support that epileptic manifestation in DCX knockdown rats only occur at adult stages. However, we cannot exclude a younger age of seizure onset in rats with larger heterotopic masses.

In order to evaluate if immature DCX knockdown rats displayed an increased susceptibility to seizures, they were injected with the convulsant agent PTZ and monitored under ECoG control. As shown in Table 2, all DCX knockdown animals displayed electrographic seizures after a single PTZ injection whereas only 2 out of 7 control rats exhibited seizures after a single PTZ injection; the remaining control animals required an additional PTZ injection to develop generalized seizures. In addition, seizures in DCX knockdown rats occurred with a significantly shorter latency after PTZ injection when compared with control rats (Table 2). Finally, lethality was also dramatically increased in the population of rats with subcortical heterotopia. A typical sample of ECoG

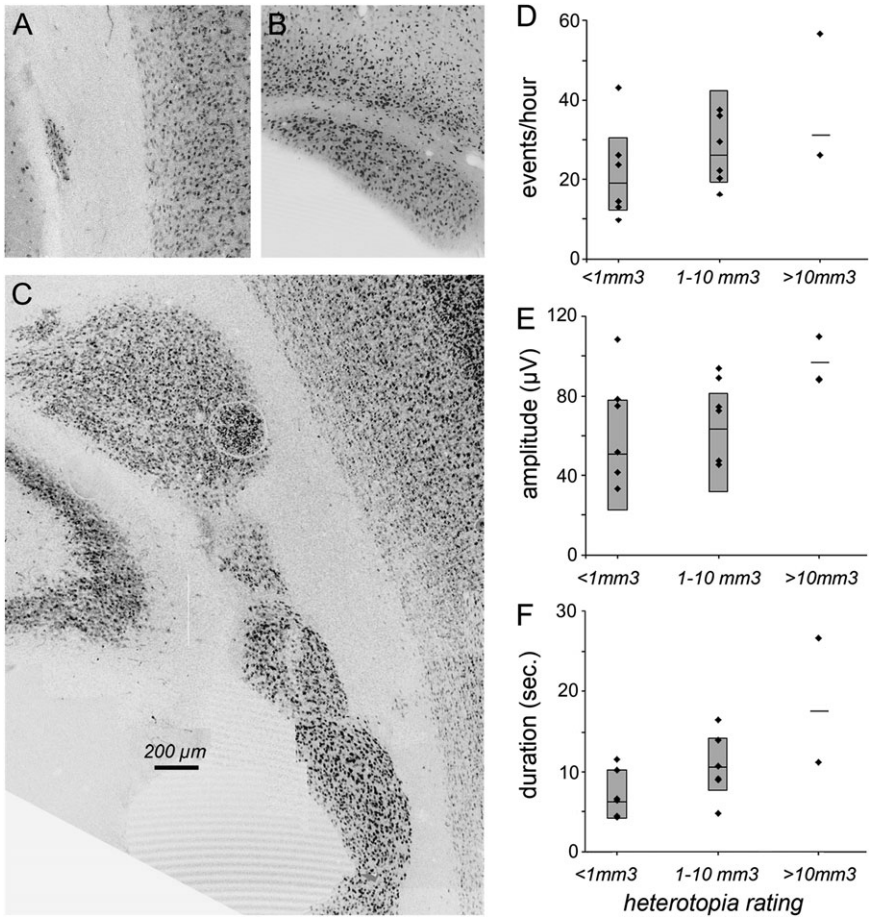

Figure 3. Histological characterization of heterotopia on adult DCX knockdown rats and correlation analysis between seizure events and heterotopia degree. Brain sections from adult-recorded rats were stained with NeuN antibodies to identify the presence of heterotopic masses of neurons in the white matter. Different degrees of alterations were observed that were classified as small (volume smaller than 1 mm^3), medium (from 1 to 10 mm^3), or large (greater than 10 mm^3). (*A–C*) Representative examples of subcortical heterotopias of different sizes. (*D–F*) Correlation analysis of heterotopia degree and frequency (*D*; Pearson 0.273, $P = 0.346$), amplitude (*E*; Pearson 0.641, $P = 0.0135$), and duration (*F*; Pearson 0.722, $P = 0.003$) of epileptic events. Each dot represents the median per animal of the events recorded 1 h/day for 3 days. Vertical box represents the median and percentiles.

recordings obtained is illustrated in Supplementary Figure 1. Analysis of PTZ effects on electroporated rats that did not display clear heterotopias revealed similar results than control animals (Table 2) additionally supporting the notion that the heterotopia rather than the manipulation of the embryos resulted in increased seizure susceptibility.

Epileptic Manifestations Get Worse with Age

The cohort of rats used in this study allowed us to identify plausible correlations between epilepsy severity and age (rat age ranged from 15 to 354 days). Interestingly, we observed a clear positive correlation between the frequency of epileptic events and the age of the animals (Fig. 5*A*). In order to segregate the contribution of age from that of the heterotopia size, we analyzed the correlation of these parameters in animal displaying small heterotopia and found again a good correlation between age and frequency of epileptiform events (Fig. 5). In addition, a positive correlation was found between the age and duration of electrographic events associated or not with motor manifestations (Fig. 5*B*). The amplitude of recorded events was not correlated with age.

Discussion

The present report demonstrates that animals with subcortical heterotopia induced by in utero RNA knockdown of DCX exhibit spontaneous motor seizures, thus reinforcing the appropriateness of this model for pathophysiological investigations of this type of cortical development disorders. Our present data show that the severity of the epileptic manifestations is correlated with heterotopia size in this animal model. Based on previous investigations, we propose that this epileptic condition emerges as a consequence of the heterotopia formation associated with secondary alterations in the cortex overlying the malformation.

Epilepsy is present in almost all patients with subcortical band heterotopia and is intractable in about 65% of them (Guerrini and Carrozzo 2001). The epileptic features of these patients are quite variable and manifested in reported cases as partial or generalized seizures (Barkovich et al. 1994; Tyvaert et al. 2008). There is no specific type of epilepsy associated with this malformation. All DCX knockdown rats analyzed in this report (displaying subcortical heterotopia) presented electroclinical manifestations compatible with partial epilepsy: animals during ECoG recorded epileptiform discharges

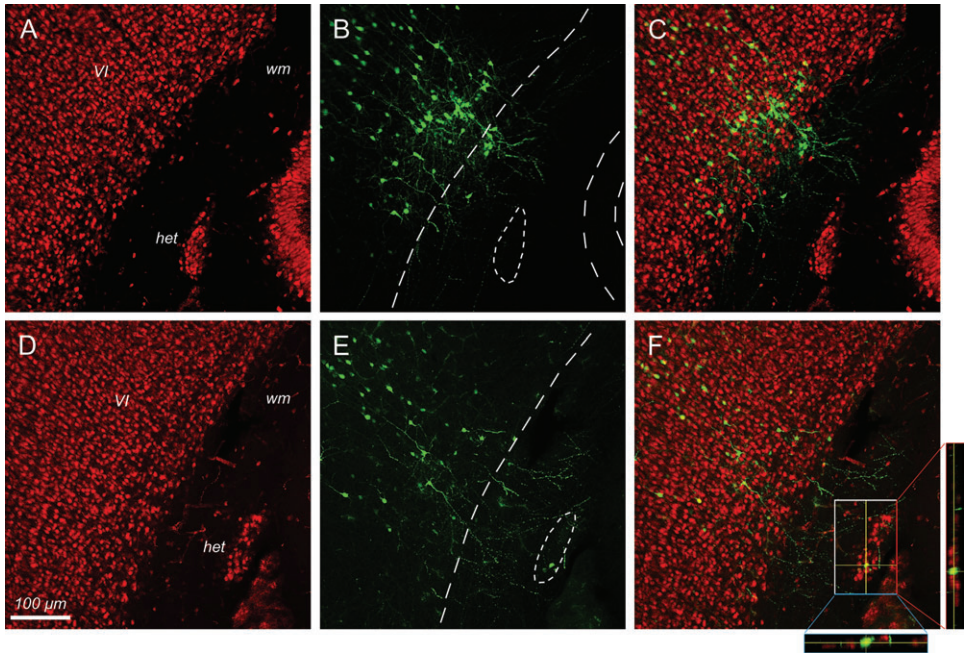

Figure 4. Confocal microphotographs showing laminar position of transfected neurons and heterotopic neuronal masses in 2 serial brain sections from a P12 DCX knockdown rat (section thickness, 100 µm). Sections were immunostained with NeuN (red in *A*, *C*, *D*, *F*) and GFP (green in *B*, *C*, *E*, *F*) antibodies. Orthogonal views in (*F*) illustrate the colocalization between NeuN (red) and GFP (green) at the level of the selected cell (image stack thickness, 45 µm). Dashed lines delineate the white matter (wm) and subcortical heterotopic masses (het).

experienced variable types of manifestations including myoclonic jerks of the jaw and tail and back clonus. DCX knockdown rats never displayed generalized seizures. Though it is quite difficult to compare ECoG features in human and rats, it can be proposed that in utero knockdown of DCX results in a good model of band heterotopia and associated epilepsy and thus provides a reliable animal model for investigating the pathophysiology and potential therapy in partial epilepsy.

It is important to note that DCX knockdown rats display epileptic features regardless the degree of cortical alteration and that even small heterotopic masses are sufficient to yield severe epilepsy, in agreement with a previous case report showing that a DCX patient suffering from resistant partial seizures presented only a subtle discontinuous subcortical heterotopia in a quite normal brain (des Portes et al. 2002). It is therefore plausible that unnoticed subtle heterotopias in human patients can be the opening cause of epileptic manifestations. Moreover, DCX knockdown rats clearly displayed a positive correlation between the size of subcortical heterotopia and the severity of the epileptic manifestations (namely the duration and amplitude of the epileptic manifestations). A previous analysis of DCX patients (Barkovich et al. 1994) already indicated that the severity of epilepsy was correlated with the severity of the pachygyria analyzed on magnetic resonance scans. However, to our knowledge, no study so far provided quantitative evidences for a positive correlation between the volume of the heterotopic band and the clinical manifestations. Based on our present observations, we propose that the severity of epilepsy and eventually other clinical manifestations in DCX patients may be dependent on the number and density of ectopic neurons. It is also important to stress that epileptic manifestations aggravate with age; thus, 2-week-old pups did not display spontaneous epileptic manifestations though they are already more susceptible to convulsant agents and therefore hyperexcitable, as compared with age-matched controls. We cannot precisely delineate the age of seizure onset; but in our series, the first epileptic manifestations were detected in 200-day-old animals. More interesting is the observation that epileptic manifestations get worse with age and that the more severe electrographic manifestations in frequency and duration were found in elder animals (about 400 days old). Interestingly, Barkovich et al. (1994) reported in a series of patients with band heterotopia that most of them had a history of progressively more complex seizure disorder as they grew older. The rationale for this clinical evolution is unclear as no apparent signs of additional brain damage induced by seizure activity were observed in stained brain sections, but it is likely that severe epileptic condition is contributing to aggravate the status of the affected animals.

Table 2
Susceptibility to PTZ-induced seizures in developing control and DCX knockdown rat pups

		Control rats (n = 7)	DCX rats with Het. (n = 4)	DCX rats with no Het. (n = 12)
Number of PTZ injections leading to seizures		2.07 ± 0.07	1	2.08 ± 0.08
Latency of PTZ-induced seizures (min after the first PTZ injection)		14.60 ± 1.2	6.15 ± 2 ($P < 0.05$)	14.62 ± 1.17
Death after PTZ	Lethality rate (number of dead rats)	22.2% (2)	100% (4)	16.6% (2)
	Time after the first injection (min)	162.5 ± 67.5	13.25 ± 4.6	148.8 ± 61.20

NOTE.—Three groups of animals were analyzed at P15: control rats, DCX knockdown rats displaying heterotopias (Het.), and DCX-electroporated rats that did not display cortical heterotopias.

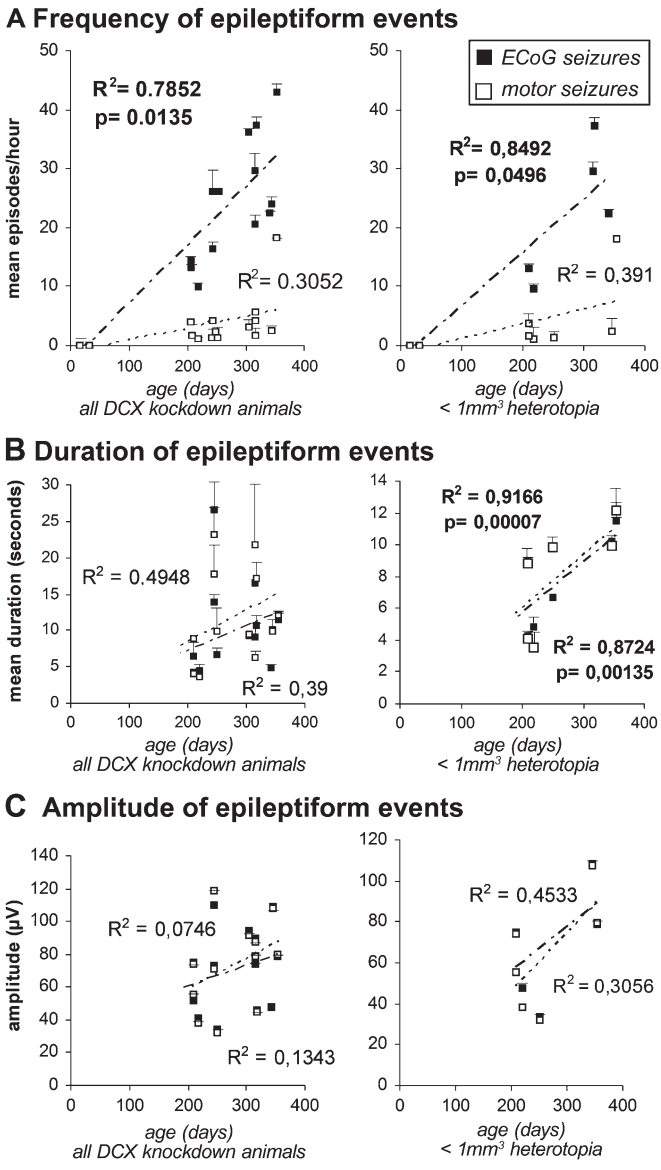

Figure 5. Epileptic events get worse with age. Analysis of correlations between age of animals at recording times and frequency (*A*), duration (*B*), and amplitude (*C*) of epileptiform events were analyzed in the whole population of DCX animals (left column) or in animals displaying heterotopias of size below 1 mm^3 (right column). Each dot represents for each animal the median (± standard error) of the events recorded for 1 h/day during 3 days. Open squares indicate electrographic events associated with motor manifestations. Black squares indicate electrographic events regardless the association or not with motor signs. Note that animals recorded during the first month of life did not display seizures and that the frequency of electrographic epileptic episodes as well as the mean duration of these events was positively correlated with age.

Knockout models of DCX failed to reproduce cortical heterotopias but associated hippocampal abnormalities (Corbo et al. 2002; Deuel et al. 2006; Nosten-Bertrand et al. 2008; Kerjan et al. 2009). These animals were either not apparently epileptic (Corbo et al. 2002) or presented epilepsy in about 40% of individuals (Nosten-Bertrand et al. 2008) or in 100% of them (Kerjan et al. 2009). Analysis so far performed concluded that epilepsy in the latter was generated within the hippocampal field before spreading to cortical structures to secondarily cause generalized tonic–clonic convulsions (Nosten-Bertrand et al. 2008; Kerjan et al. 2009). Regarding the human pathology, these models have a limited impact in the field as there are only 3 published cases reporting hippocampal alterations (mainly hypoplastic features) in human fetus with DCX mutations (Kappeler et al. 2007). A more reliable model of band heterotopia is the *Tish* rat that displays spontaneous seizures described as twitching of the face and paws and turning of the animal to one side with in some cases signs of secondary epileptic generalization (Chen et al. 2000). The gene responsible for this condition remains unknown, but the genesis of the heterotopia is linked to an abnormal proliferation of neuronal precursors (Lee et al. 1998). *Tish* rat cannot therefore be considered as a model of neuronal migration disorders. RA-GEF-1 KO (Bilasy et al. 2009) and HeCo mice (Croquelois et al. 2009) models have been more recently described that also display massive subcortical band heterotopia. The genetic origin and mechanism of genesis of the band heterotopia have not been elucidated yet. Although these mice showed an increased tendency to develop convulsant-induced seizures, they did not display spontaneous epileptic seizures (Bilasy et al. 2009; Croquelois et al. 2009), but more should be done to elucidate this issue. Tish, HeCo, and RA-GEF-1 models of cortical malformation rather constitute orphan models that interest would be reinforced after the identification of their genetic basis and the discovery of patients with similar syndromes. In contrast, DCX knockdown model offers the advantage of a band heterotopia formed as a clear migration defect, originated by the same gene breakdown involved in human double cortex. We demonstrate here that this alteration is associated with the development of spontaneous epileptic manifestations with features reminiscent to that described in human patients with DCX mutation. It provides an exceptional, if not the only one, reliable model so far described for this pathological condition. Thus, recent investigations from our laboratory (Ackman et al. 2009) have shown a strong reduction in the frequency of spontaneous excitatory postsynaptic currents (EPSCs) and inhibitory postsynaptic currents in ectopic neurons of DCX knockdown rats as compared with normal layer 2/3 (L2/3). Importantly, the presence of ectopic neurons also impacts the activity of nontransfected overlying L2/3 neurons: though they migrated to their appropriate layer, they displayed an important alteration in the excitatory/inhibitory balance toward excitation due to a strong increase in the frequency of spontaneous EPSCs as compared with neurons in normal L2/3. Accordingly to these observations, both subcortical band heterotopia and overlying cortex contribute to epileptic manifestations, in agreement with previous observations on human patients using ECoG–functional magnetic resonance imaging (Tyvaert et al. 2008). Future investigations are required to clarify which structure is the epilepsy generator: the malformation, the surrounding tissue, or both.

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Supplementary Material

Supplementary materials can be found at: http://www.cercor.oxfordjournals.org/.

Notes

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