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ORIGINAL ARTICLE

Prenatal exposure to the CB_1 and CB_2 cannabinoid receptor agonist WIN 55,212-2 alters migration of early-born glutamatergic neurons and GABAergic interneurons in the rat cerebral cortex

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Abstract

The endocannabinoid system, composed of cannabinoid receptors, endocannabinoids, and synthesis and degradation enzymes, is present since early stages of brain development. During this period, the endocannabinoid system is involved in the regulation of neural progenitor proliferation and specification as well as the migration and differentiation of pyramidal neurons and interneurons. Marijuana consumption during pregnancy represents a serious risk in relation to the fetal brain development since Δ^9-tetrahidrocannabinol, the main active compound of *cannabis*, can reach the fetus through placenta and hemato-encephalic barrier. Cohort studies performed on children and adolescents of mothers who consumed marijuana during pregnancy reported cognitive and comportamental abnormalities. In the present study, we examined the expression of the cannabinoid receptor CB_1R during corticogenesis in radially and tangentially migrating post-mitotic neurons. We found that prenatal exposure to WIN impaired tangential and radial migration of post-mitotic neurons in the dorsal pallium. In addition, we described alterations of two transcription factors associated with proliferating and newly post-mitotic glutamatergic cells in the dorsal pallium, Tbr1 and Tbr2, and disruption in the number of Cajal–Retzius cells. The present results contribute to the knowledge of neurobiological substrates that determine neuro-comportamental changes that will persist through post-natal life.

Keywords: cannabinoid, CB_1 receptor, developing cerebral cortex, post-mitotic migrating neurons, prenatal exposure.

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Cannabis, the most commonly abused illicit drug by pregnant women, is increasingly being recognized for both its toxic and therapeutic properties (e.g., treatment of the emotional and neurodegenerative disorders and as an analgesic in neuropathic pain; Pertwee 2009). Research on the harmful and healthful effects of cannabis use remains important since controversy regarding its therapeutic use and legalization still exists. On the other hand, little is known about the effects of cannabinoid drugs on the fetus when consumed during pregnancy. Therefore, possible teratogenic effects of endocannabinoid (eCB) system-based therapies in pregnant women and long-term exposure to cannabis need to be taken into consideration. Exogenous cannabinoids can be transferred from the mother to the offspring through the blood-placental barrier during gestation and affect fetal neuronal development (Hutchings *et al.* 1989). Therefore, manipulations of the eCB system during critical stages of brain development may have persistent neurobehavioral consequences. Several longitudinal cohort studies in children prenatally exposed to marijuana revealed a significant impairment of higher cognitive functions, such as impaired attention, learning, executive function, working memory, (Smith *et al.* 2006; El Marroun *et al.* 2011; Huizink, 2013), and psychiatric disorders (Jutras-Aswad *et al.* 2009; Mathews *et al.*, 2014). Adverse effects of exposure to cannabinoids during brain development have also been dem-

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Abbreviations used: CB_1R, cannabinoid receptor type 1; CB_2R, cannabinoid receptor type 2; CCK, cholecystokinin; CP, cortical plate; DCX, doublecortin; eCB, endocannabinoid; FAAH, fatty acid amine hidrolase; GE, ganglionic eminence; IZ, intermediate zone; MZ, marginal zone; PP, preplate; SP, subplate; SVZ, subventricular zone; THC, Δ^9-tetrahidrocannabinol; VZ, ventricular zone; WIN, WIN 55,212-2.

onstrated in animal models. Prenatal exposure to the cannabinoid receptors agonist WIN 55,212-2 (WIN) or Δ^9-tetrahidrocannabinol causes learning and memory disruption in rat adult offspring. Moreover, this effect was proven to be associated with permanent alterations in cortical glutamatergic neurotransmission, hippocampal long-term potentiation, and glutamate release (Mereu *et al.* 2003; Antonelli *et al.* 2005). Even thought there are several studies that demonstrate the long-term consequence of prenatal cannabinoid exposure, the effect on embryonic brain is unknown.

The eCBs consists mainly of two types of cannabinoid receptors, type one (CB_1R) and type two (CB_2R) receptors, their endogenous lipid ligands, the eCB, and the enzymatic machinery for their synthesis and degradation (De Petrocellis and Di Marzo 2009). During neuronal development, CB_1R is expressed in neural progenitors, where receptor levels increase throughout neuronal differentiation and progressively localize to developing axonal projections (Berghuis *et al.* 2007; Mulder *et al.* 2008). During early brain development, CB_1R not exert the classical neuromodulatory role of the eCB system in the mature adult brain. Instead, during corticogenesis, CB_1R regulates several aspects of neural development, including neural progenitor proliferation, radial and tangential migration of pyramidal cortical neuron and interneurons, and axonal pathfinding and synaptogenesis (Berghuis *et al.* 2005, 2007; Mulder *et al.* 2008; Trazzi *et al.* 2010; Wu *et al.* 2010; Diaz-Alonso *et al.* 2012).

Since the cerebral cortex is a major site for processing of cognition and memory and cortical glutamate is involved in the regulation of cognitive functions, it could be speculated that prenatal exposure to WIN, might lead to an inappropriate neuronal development that could represent the substrate for the deficit of learning and memory functions observed in children born to women who consumed marijuana during pregnancy. Thus, in view of the evidence for a detrimental effect of prenatal cannabinoid exposure on cognitive and neurobehavioral process and for the role of eCB system in brain development, the aim of this study was to determine the effects of gestational exposure to WIN on neural progenitor proliferation, neuronal migration and differentiation on embryonic rat cerebral cortex. This work provides the first evidence on the effects of gestational cannabinoid exposure on early-born glutamatergic neurons and GABAergic interneurons migration and glutamatergic neurons differentiation.

Materials and methods

Ethics statement

All procedures for the care and the use of experimental animals were approved by the *Institutional Committee for the Care and Use of Laboratory Animals* of School of Medicine, University of Buenos Aires (CICUAL) and were in strict accordance with the *recommendations in the Animal Research: Reporting of In Vivo Experiment (ARRIVE) guidelines* for the care and use of laboratory animals. All efforts were made to minimize animal suffering and discomfort and the number of animals used was the minimum necessary for meaningful interpretation of data. All surgery was performed under ketamine–xylazine anesthesia.

Animals and exposure conditions

Primiparous Wistar female rats (School of Pharmacology and Biochemistry of the University of Buenos Aires) weighting 240–260 g were mated. The day on which sperm were present was designated as the embryonic day 0.5 (E0.5). Pregnant rats were randomly assigned to two groups per embryonic day (n = 4/5 per group): control (C) and WIN-exposed (WIN). Pregnant rats received a daily subcutaneous injection of WIN 55-212,2 (0.75 mg/Kg) or vehicle (0.3% Tween 80/saline at the volume of 1.0 mL/Kg) in the dorsal neck from gestational day 5 to 12, 14, 16 or 20, respectively. This dose was chosen on the basis of a previous study showing that prolonged prenatal exposure to a higher WIN dose (1 mg/kg) significantly affected reproduction parameters such as dam weight gain, as well as litter size at birth and post-natal mortality (Mereu *et al.* 2003; Shabani *et al.* 2011). One fetus per litter from different litters per treatment group was used in each experiment.

Reproduction data

Body weights of the dams from gestational day 0 to 20 and litter size and prenatal mortality (fetal resorption per litter) were recorded.

Fixation of embryonic brains

Time-mated pregnant rats were anesthetized (ketamine–xylazine, 70 mg/kg–10 mg/kg, i.p.), and the embryos were obtained by hysterectomy at different embryonic stages. Whole heads (E12.5, 13.5, and E14.5) or isolated brains (E15.5, E16.5, and E20.5) were removed under a dissecting microscope and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer pH7.4 at 4°C overnight. Fixed E12.5, E13.5, E14.5, E16.5, and E20.5 whole brains were cryoprotected in 30% sucrose in 0.1 M phosphate buffer saline pH 7.4, frozen in dry ice, and store at −80°C until further processing. Coronal cryostat sections (20 μm) were mounted on silane-coated slides, air dried, and store at −20°C.

Immunohistochemistry

Mounted sections were incubated in blocking solution (5% normal goat serum and 0.5% Triton X-100 diluted in 0.1 M phosphate buffer saline). Incubation with primary antibody was performed overnight at 4°C. For immunofluorescence, secondary antibodies, goat anti-mouse, or anti-rabbit conjugated with fluorescein isothiocyanate (FITC) or Texas Red and goat anti-guinea pig conjugated with FITC (1 : 400, Vector Laboratories, Burlingame, CA, USA) were used. Sections were later counter-stained with Hoechst 33342 (Sigma, St Louis, MO, USA) to label nuclei and coverslipped with anti-fading mounting medium Fluoroshield (Sigma). The primary antibodies used were as follows: rabbit anti-CB_1R (1 : 1000; C-term; Cayman Chemicals, An Arbor, MI, USA); rabbit anti-Tbr1 (1 : 1000; Abcam, Cambridge, MA, USA); rabbit anti-Tbr2 (1 : 1000; Chemicon International, Inc, Temecula, CA, USA); guinea pig anti-doublecortin (DCX; 1 : 4000; Chemicon International, Inc.); mouse anti-Reelin (1 : 1000; clone G10; Millipore, Temecula, CA, USA); mouse anti-Ki-67 (1 : 800; clone Ki-S5; Chemicon International, Inc.); mouse anti-Nestin (1 : 4000; clone Rat-401; Chemicon International, Inc.),

rabbit anti-GABA (1 : 1000; Sigma Aldrich, St Louis, MO, USA). Experiments were carried out at least in triplicate.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling analysis

The number of cells undergoing apoptosis was analyzed by Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling analysis (TUNEL) using the ApopTag Plus Fluorescein *In Situ* Apoptosis Detection Kit (Chemicon International, Inc.) according to the manufacturer's instructions. For negative controls, the TdT enzyme was omitted. For positive controls, the slices were incubated with 10 U/mL DNAse I prior to treatment with the TUNEL reaction to induce free 3′-OHs on DNA.

Microscopy and images acquisition

Images were acquired on an *Axiolab* epifluorescence microscope (Carl Zeiss, Oberkochen, Germany) with a *Q-Color3* CCD camera (Olympus, Tokyo, Japan) or a *FluoView FV1000* Confocal Laser Scanning System (Olympus). Counting and morphometry were performed using Image Pro PLUS 4.5 (Media Cybernetics, Warrendale, PA, USA) and Image J (NIH, http://rsb.info.nih.gov/ij/) softwares.

Cell quantification

We used four to eight coronal serial no-consecutive sections from each brain per prenatal treatment condition and per experiment. Embryonic cerebral cortex layers were identified by their discrete cell densities as visualized by Hoechst 33342 counter-staining. Cells were counted from a 500 μm wide profile of the dorsal pallium in sections through the presumptive medial pre-frontal cortex on least four/five different animals.

Quantification of the orientation of DCX+ cells

For the analysis of the orientation of migrating cells in the developing cerebral cortex, DCX+ cells were identified in the ventricular zone (VZ)/subventricular zone (SVZ) and the migration angle was measured between the virtual vertical lines positioned at 0 or 90° and the main leading process. Cells that deviated less than 25° from radial lines were considered as radially oriented and those that deviated more than 25° were designated as tangentially oriented.

Statistical analyses

In each experiment, for each prenatal treatment condition, we used samples collected from one or two embryo from a given litter. We used four to five litters of rats from each prenatal treatment condition. Thus, we used data from four to five individuals (n = 4/5) per prenatal treatment condition. Differences between prenatal treatment conditions were analyzed for statistical significance by using two-tailed Student's t-test. All data are expressed as means ± SEM and $p < 0.05$ was considered significant. Data were subjected to statistical analyses using GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA, USA).

Drugs

(R)-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1, 2,3-de)-1,4-benzoxazin 6-yl]-1-napthalenylmethanone (WIN 55, 212-2) were purchased from Sigma.

Results

CB_1R is expressed in migrating post-mitotic neurons during cerebral cortex development

During corticogenesis, early-born post-mitotic pyramidal neurons originate from the proliferative VZ/SVZ of the dorsal pallium and migrate radially to populate, first, the preplate (PP) and then the cortical plate (CP) in a layer-specific manner In contrast, post-mitotic cortical interneurons originate from the VZ of the ganglionic eminence (GE) and follow tangential migratory routes to populate the cerebral cortex (Molyneaux *et al.* 2007). Migrating post-mitotic neurons are identified by expression of the marker DCX, a microtubule-associated protein that is required for migration and correct lamination in the developing cerebral cortex (Francis *et al.* 1999). To assess whether CB_1R is expressed in migrating post-mitotic neurons, embryonic telencephalic sections were immunostained for specific antibodies against CB_1R and DCX. At early stage of corticogenesis (E12.5 and E13.5), most migrating post-mitotic neurons in the PP expressed CB_1R (Fig. 1a–f). CB_1R was also detectable in tangentially migrating post-mitotic neurons (Fig. 1d–f). Also CB_1R was expressed in radially migrating post-mitotic neurons in the VZ (Fig. 1a–c). At mid stage of corticogenesis (E14.5 to E16.5), we found abundant CB_1R expression in the DCX+ cells in the mantle zone of GE and, occasionally, in the proliferative zone of GE (Fig. 1g–i). At E16.5 CB1 was expressed in post-mitotic migrating neurons in the marginal zone (MZ), CP and subplate (SP), and in developing axons of pyramidal neurons in the intermediate zone (IZ; Fig. 1j–l). At this stage, CB_1R was expressed in radial post-mitotic neurons in SVZ near the subpallium-pallium boundary (Fig. 1m–o).

Prenatal exposure to WIN did not affect reproductive parameters

Overall, one-way ANOVA demonstrated that dam weight gain during pregnancy (mean% dam weight gain ± SEM from embryonic day 0 to 20) and litter size at all embryo stage analyzed (mean number of embryos ± SEM) were not significantly affected by prenatal exposure to 0.75 mg/Kg WIN (Table 1). In addition, prenatal exposure to WIN did not influence resorption rate in pregnant rats (data not shown). Since the used dose of WIN did not produce malformation or apparent signs of toxicity nor significantly altered gestational parameters, it could be assumed that the alteration observed in the developing cerebral cortex of WIN-exposed embryos were not because of indirect effects of the agonist, such as mother malnutrition.

Prenatal exposures to WIN affect de number of radial and tangential post-mitotic migrating neurons during the development of the cerebral cortex

Post-mitotic migrating neurons exhibits a main leading process extending from the soma, and the angle at which

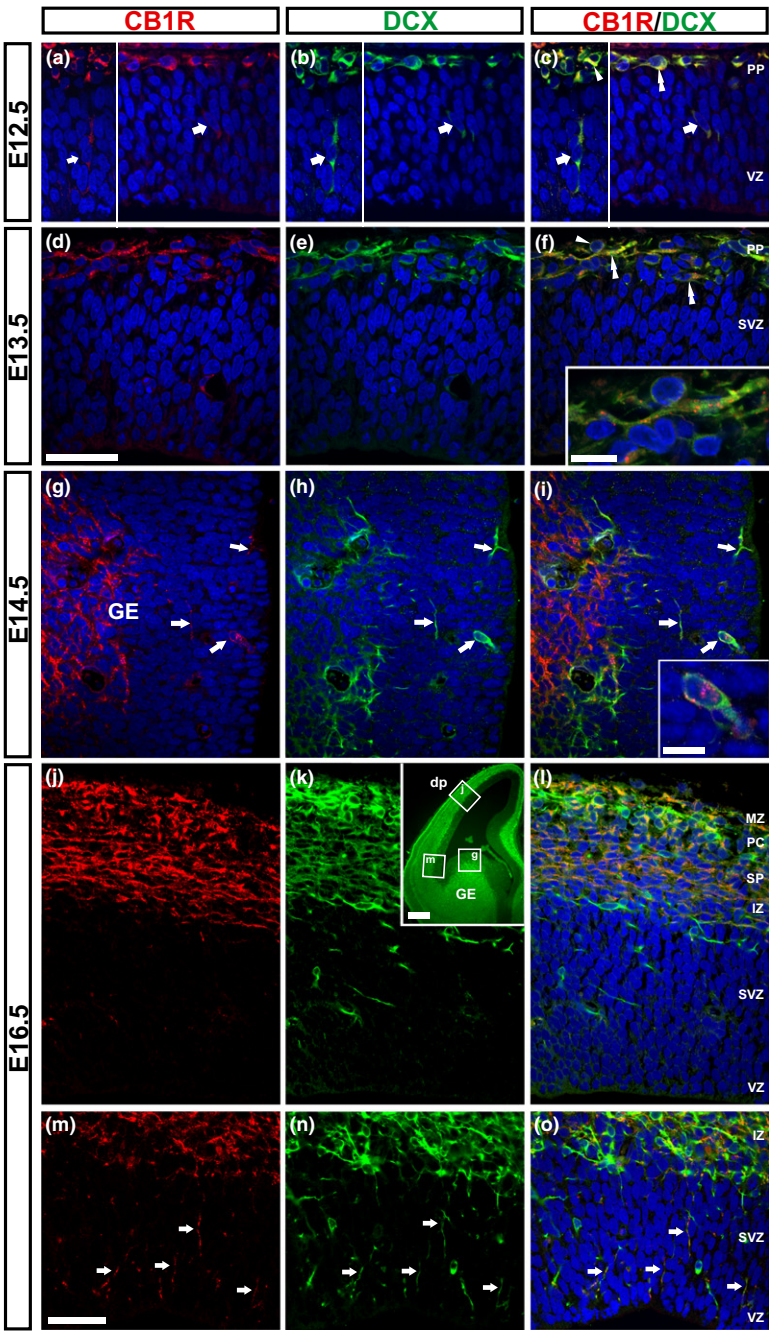

Fig. 1 Temporal CB_1R expression in migrating post-mitotic neurons during corticogenesis. (a–f) Representative confocal images of CB_1R and doublecortin (DCX) expression in coronal dorsal pallium sections during early stages of corticogenesis, E12.5 (a–c) and E13.5 (d–f). Arrow indicates CB_1R expression in radial post-mitotic migrating neurons in ventricular zone (VZ), and double-arrowheads indentify CB_1R expression in tangentially post-mitotic migrating neurons. Higher magnification image in f show a tangential oriented post-mitotic neurons expressing the receptor. (g–i) Representative confocal images of CB_1R and DCX expression in ganglionic eminence (GE) at E14.5. Arrow indicates CB_1R expression in GE subventricular zone (SVZ). Higher magnification image in (i) show a migrating post-mitotic neurons expressing the receptor. (j–o) Representative confocal images of CB_1R and DCX expression in coronal dorsal pallium sections during mid stages of corticogenesis, at E16.5. Inset in k shown DCX expression in a telencephalic coronal section at E16.5. White box denotes localization of representative images. Scale bars: 40 μm in a; 10 μm in (f and i) inset; 200 μm in (k) inset.

leading process are formed determines the possible directions that can be followed by migrating cells (Martini *et al.* 2009). Given the angle of this process, we categorized the DCX+ cells in radially or tangentially migrating neurons. Labeling was abundant throughout the cortical mantle, including a stream of horizontally oriented cells at the level of the lower IZ and radial cells in the VZ/SVZ. The cells whose main neurite determined an angle less than 25° with respect to a virtual vertical line were designated as neurons in radial migration process, and the cells whose main neurite determined an angle greater than 25° were certain designated as neurons in tangential migration (Fig. 2b). Given that, at E16.5, the exact fraction of DCX+ cells in the dorsal pallium is not possible to estimate in densely packed layers such as the MZ, SP, and upper IZ, we only quantified the DCX+ cells in the VZ/SVZ (Fig. 2a). There was a significant increase in the number of post-mitotic neurons, both in tangential orientation (C: 8.63 ± 1.18 *vs.* WIN: 20.55 ± 2.44 cells/600 μm wide bin; $p = 0.0292$) as well as in radial orientation (C: 32.02 ± 2.99 *vs.* WIN:

Table 1 Effect of prenatal WIN treatment on dam weight gain during pregnancy and litter size

		litter size			
Group	Dam Weight gain %	E12.5	E14.5	E16.5	E20.5
Control	43.32 ± 4.36	10.0 ± 2.0	11.8 ± 0.3	10.8 ± 0.9	13.0 ± 1.2
WIN	46.95 ± 4.49	11.5 ± 0.9	13.0 ± 1.1	11.4 ± 2.2	10.8 ± 1.9

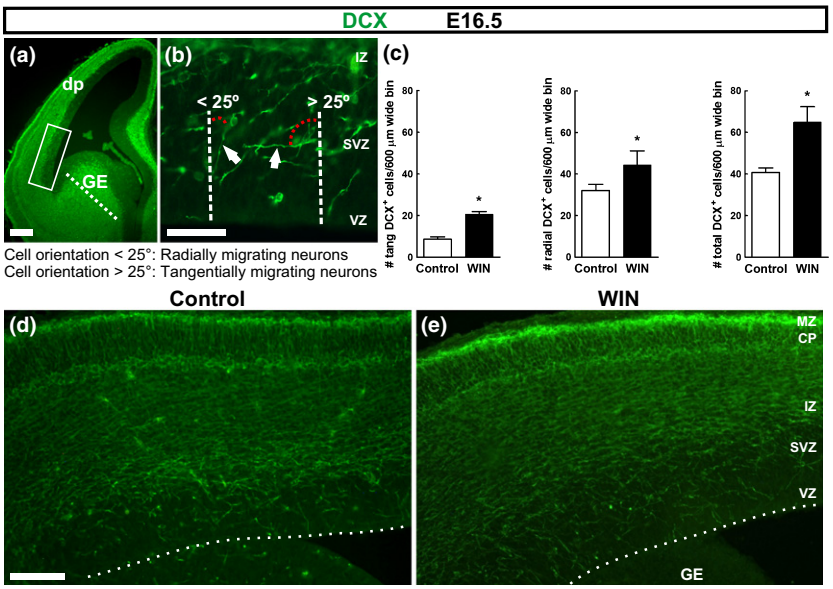

Fig. 2 Prenatal WIN-exposure impaired the number of radial and tangential post-mitotic migrating neurons in the ventricular zone (VZ)/subventricular zone (SVZ) of embryonic cerebral cortex. (a) Double-cortin (DCX) expression in a telencephalic coronal section at E16.5. White box denotes quantification section. (b) Schematic showing the criteria for the classification of the orientation of DCX+ cells in cortical sections. The migration angle was measured between the virtual vertical/transversal lines positioned at 0° or 90° and the post-mitotic neuron main leading process. Cells that deviated less than 25° from radial lines were considered as radially oriented; and those that deviate more than 25° were designated as tangentially oriented. (c) Bar graphs show quantification of tangential, radial and total post-mitotic migrating neurons in the VZ/SVZ in the developing cerebral cortex (*$p < 0.05$ Student's *t*-test, error bars represent SEM, $n = 4$ for control and WIN). (d, e) Telencephalic coronal sections immunostained for DCX in control (d) and WIN-exposed (e) embryos at E16.5. Dashed lines indicate the ventricular surface. Scale bars: 200 μm in a; 50 μm in b; 100 μm in d and e.

44.34 ± 6.83; $p = 0.0428$) in WIN-exposed embryos (Fig. 2c). The total number of cells in the VZ/SVZ of the WIN-exposed embryos also presented a significant increase (C: 40.66 ± 2.27 *vs.* WIN: 64.89 ± 7.41; $p = 0.0148$) (Fig. 2c).

Prenatal exposure to WIN increased the number of migrating GABAergic interneurons through the cortical marginal zone

At E16.5 migrating interneurons follow two distinct migratory streams into the dorsal pallium: one superficial, within the MZ and the SP, and the other, in the IZ and SVZ (Tanaka *et al.* 2003). In order to evaluate the effect of prenatal exposure to WIN on migrating GABAergic interneurons, both in the superficial and deep migratory streams, we performed an immunofluorescence staining for GABA on thelencephalic coronal sections of E16.5 control and WIN-exposed embryos (Fig. 3a–b). We quantified the number of $GABA^+$ cells separately in each layer: MZ, SP, CP, and SVZ/IZ in a region of the medial dorsal pallium. The embryos exposed to WIN showed a statistically significant increase in the number of $GABA^+$ cells in the MZ (C: 141.90 ± 17.89 *vs.* WIN: 213.20 ± 10.46; $p = 0.0379$). No significant differences were found in any of the other layers: CP (C: 33.74 ± 9.15 *vs.* WIN: 34.27 ± 11.33; $p = 0.8056$), SP (C: 79.68 ± 17.34 *vs.*

WIN: 70.80 ± 12.82; $p = 0.6377$) and IZ/SVZ (C: 472.00 ± 20.88 *vs.* WIN: 428.50 ± 8.77; $p = 0.0794$) or in the total number of GABA$^+$ cells (C: 727.4 ± 15.64 *vs.* WIN: 746.80 ± 28.85; $p = 0.4574$) (Fig. 3c).

Prenatal exposure to WIN did not affect cellular proliferation and programmed cell death in the developing cerebral cortex

The increase in the number of DCX+ migrating cells in the VZ/SVZ observed in WIN-exposed embryo could be because of an alteration in the proliferation and/or in programmed cell death. We tested whether the proliferation of VZ and SVZ cortical progenitors was affected by prenatal exposure to WIN at three representative time points: E12.5, E14.5, and E16.5. Coronal sections of the embryonic thelencephalon were immunostained for nuclear cell proliferation antigen Ki67 and the number of mitotic cells at the VZ surface, that represent radial glial cell divisions, and mitotic cells at the SVZ, that represent intermediate progenitor cell divisions, were evaluated. The number of ventricular and adventricular mitoses did not differ between control and WIN-exposed embryos at any stage evaluated, suggesting the possibility that the effects seen were attributable to changes in migration and not proliferation (Fig. 4a–g).

Previous reports have suggested that exposure to WIN can down-regulate pro-apoptotic and up-regulate non-apoptotic molecules in the cerebral cortex of adult rodents (Alvaro-Bartolome *et al.* 2010). Therefore, in order to exclude the possibility that prenatal exposure to WIN induced an alteration in programmed cellular death, a TUNEL reaction was performed on telencephalic sections to label apoptotic cells. The number of TUNEL+ cells was quantified in the dorsal pallium at E16.5 (Fig. 4h–j) and E20.5 (data not shown). Few apoptotic cells were detected in both control and WIN treated groups but no significant difference in the number of TUNEL+ cells were detected in the developing cerebral cortex.

Prenatal exposure to WIN affected the differentiation of glutamatergic neurons in the developing cerebral cortex

The glutamatergic neuronal lineage progresses along a differentiation pathway that is marked by sequential changes in the expression of transcription factors: the intermediate progenitor cells committed to glutamatergic fate express the T-box transcription factor Tbr2 while the early post-mitotic glutamatergic pyramidal neurons in layer VI and SP express the T-box transcription factor Tbr1 (Englund *et al.* 2005). In order to evaluate if neuronal glutamatergic differentiation was impaired in prenatal WIN-exposed embryos we performed immunofluorescence for Tbr2 and Tbr1 on telencephalic coronal sections at E12.5, E14.5, E16.5, and E20.5. The number of cells that expressed Tbr2 or Tbr1 was quantified in 500 μm wide bins that stretched from the ventricle to the pial surface in the dorsal pallium of control and WIN-exposed embryos. The analysis of the results showed that prenatal exposure to WIN increased the number of intermediate progenitors Tbr2+ at E12.5 (C: 49.89 ± 2.58 *vs.* WIN: 64.58 ± 5.78, $p = 0.0444$), E14.5 (C: 416.2 ± 24.61 *vs.* WIN: 471.30 ± 15.77, $p = 0.0259$), E16.5 (C: 743.30 ± 22.65 *vs.* WIN: 995.00 ± 37.33, $p = 0.049$) and E20.5 (C: 657.90 ± 28.70 *vs.* WIN: 1117.00 ± 65.37, $p = 0.0033$) (Fig. 5a–i). On the other hand, For the contrary we found a significant decreased in the number of Tbr1+ cells in

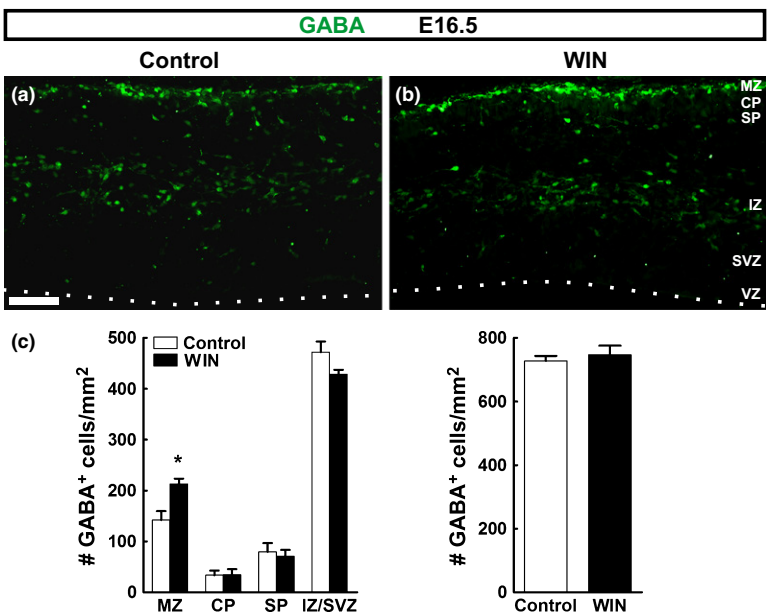

Fig. 3 Prenatal exposure to WIN increased the number of GABAergic interneurons in the marginal zone (MZ) of the dorsal pallium. (a, b) dorsal pallium coronal sections immunostained for GABA in control (a) and WIN-exposed (b) embryos at E16.5. Dashed lines indicate the ventricular surface. (c) Bars graph show quantification of GABA+ cells in the MZ, cortical plate (CP), subplate (SP), intermediate zone (IZ)/subventricular zone (SVZ) expressed as the number of cells per mm^2 and quantification of the total number of GABA+ cells in all layers. (*$p < 0.05$, Student's *t*-test, error bars represent SEM, $n = 5$ for control and WIN). Scale bar: 100 μm in a and b.

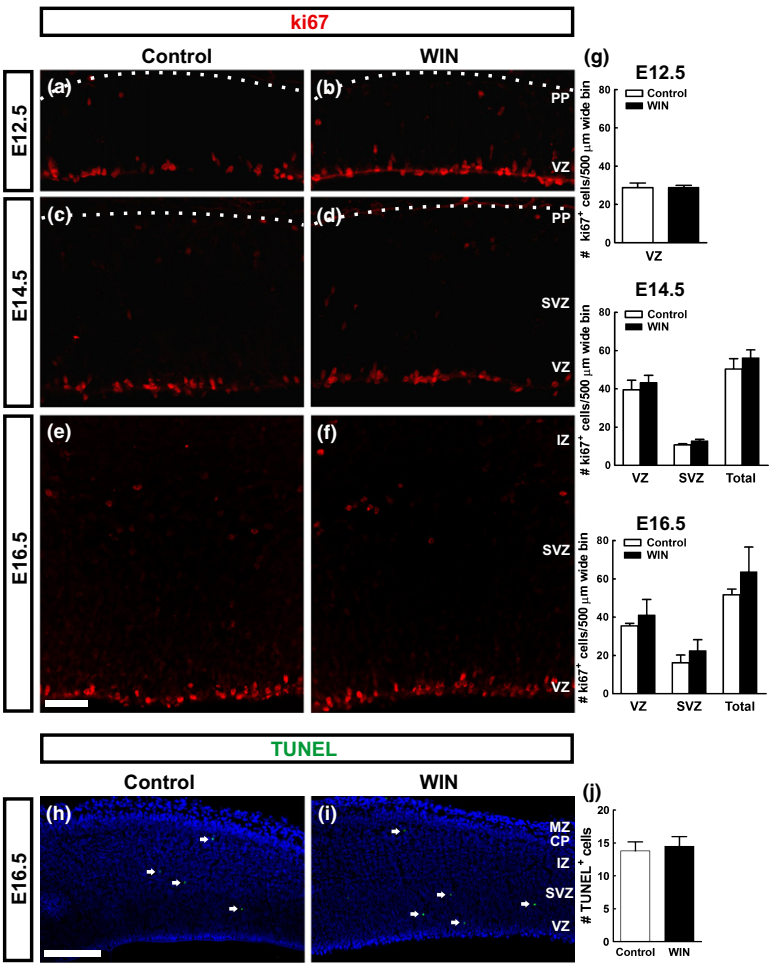

Fig. 4 Prenatal exposure to WIN did not affect the number of cells proliferating in the ventricular zone (VZ) and subventricular zone (SVZ) and the number of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling analysis (TUNEL)+ cells of the dorsal pallium. (a–f) Telencephalic coronal sections immunostained for Ki67 in control (a, c and e) and WIN-exposed (b, d and f) embryos at E12.5, E14.5, and E16.5. (g) Bars graph show quantification of ki67+ cells in VZ and SVZ and total number ($p > 0.05$, Student's *t*-test, error bars represent SEM, $n = 4/5$ for control and WIN). (h and i) Telencephalic coronal sections staining for TUNEL reaction in control and WIN-exposed embryos at E16.5. (j) Bars graph show quantification of TUNEL+ cells ($p > 0.05$, Student's *t*-test, error bars represent SEM, $n = 3$ for control and WIN). Scale bars: 50 μm in (a–f); 200 μm in (h and i).

WIN-exposed embryos at E12.5 (C: 40.75 ± 2.75 *vs.* WIN: 24.83 ± 2.17, $p = 0.0004$) and E14.5 (C: 241.60 ± 22.58 *vs.* WIN: 150.80 ± 7.48, $p = 0.0372$). However, the number of Tbr1+ cells on WIN-exposed embryos at E16.5 (C: 768.30 ± 100.20 *vs.* WIN: 661.70 ± 42.06, $p = 0.4889$) and E20.5 (C: 1052 ± 12.02 *vs.* WIN: 1060 ± 45.09, $p = 0.884$) reached the values of control embryos (Fig. 6a–g).

Effects of prenatal exposure to WIN on Cajal–Retzius cells and radial glial scaffold in the developing cerebral cortex

CB_1R is expressed in Cajal–Retzius cells that synthesize and secrete Reelin, which is essential to guide early-born post-mitotic pyramidal neurons along radial glial fibers (Hartfuss *et al.* 2003; Vitalis *et al.* 2008). In view of the possible disrupted neuronal migration in the prenatal WIN-exposed embryos we explored the effects of prenatal exposure to WIN on Cajal–Retzius cells. At E14.5, quantification of Reelin+ cells revealed an increase in Cajal–Retzius cells in WIN-exposed embryos. At E16.5, the number of Reelin+ cells in the cortical MZ was similar between control and WIN-exposed embryos (Fig. 7a–e).

Radial glial cells function as both neural progenitors in the VZ and scaffolding for neuronal migration during corticogenesis (Campbell and Gotz 2002). We analyzed the organization of the radial glial scaffold at E14.5 and E16.5 using antibodies against Nestin, an intermediate filament protein widely used as a radial glial cell marker. No significant differences were detected in the relative area neither of Nestin+ fibers nor in morphology or arrangement of radial glial processes between control and WIN-exposed embryo (Fig. 7f–j).

Discussion

The long-term effects of prenatal exposure to a cannabinoid agonist on neuro-behavior in the offsprings are well documented. However, little is known about the effects of prenatal cannabinoid exposure on the embryonic cerebral cortex. This study provides new evidence on how prenatal exposure to a relatively low dose of a synthetic cannabinoid agonist can produce significant effects on developmental processes of the neocortex, such as neuronal migration and differentiation.

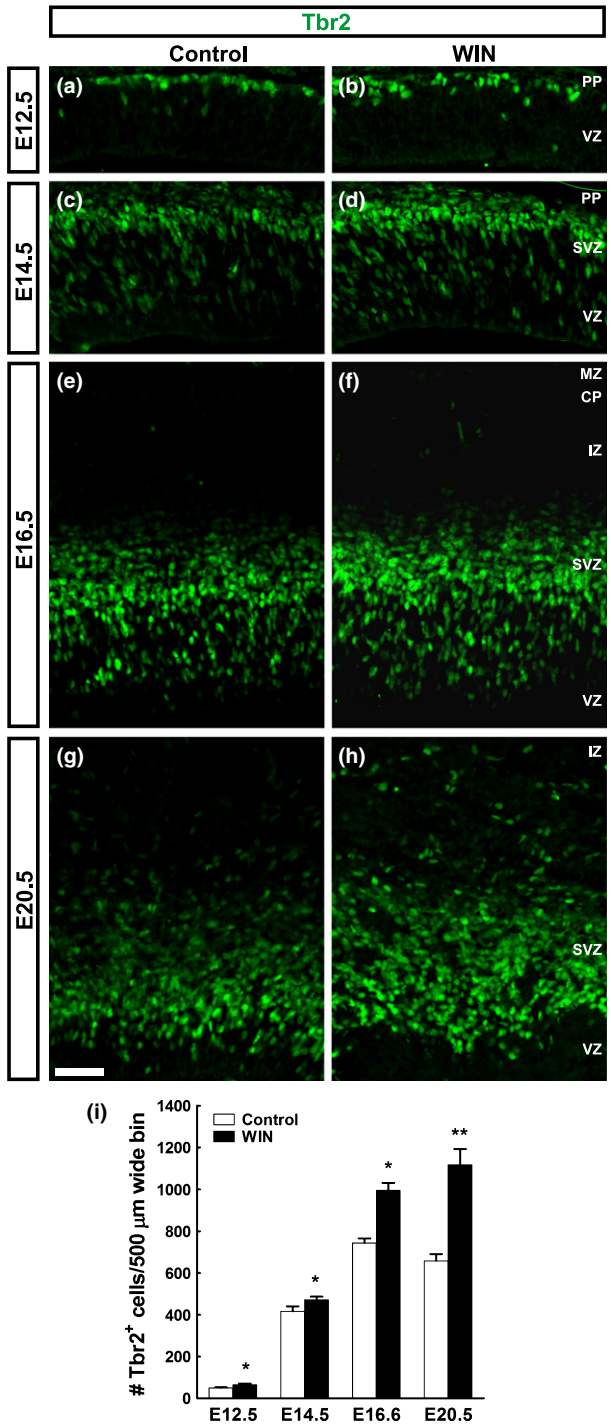

Fig. 5 The number of glutamatergic intermediate progenitors was increased in the developing cerebral cortex of WIN-exposed embryo. (a–h) Dorsal pallium coronal sections immunostained for Tbr2 in control (a, c, e, and g) and WIN-exposed (b, d, f, and h) embryos at E12.5, E14.5, E16.6, and E20.5. (i) Bars graph show quantification of Tbr2+ cells in the developing cerebral cortex of control and WIN-exposed embryos. (*$p < 0.05$, **$p < 0.005$, Student's *t*-test, error bars represent SEM, $n = 4$ for control and WIN). Scale bar: 100 μm in (a–h).

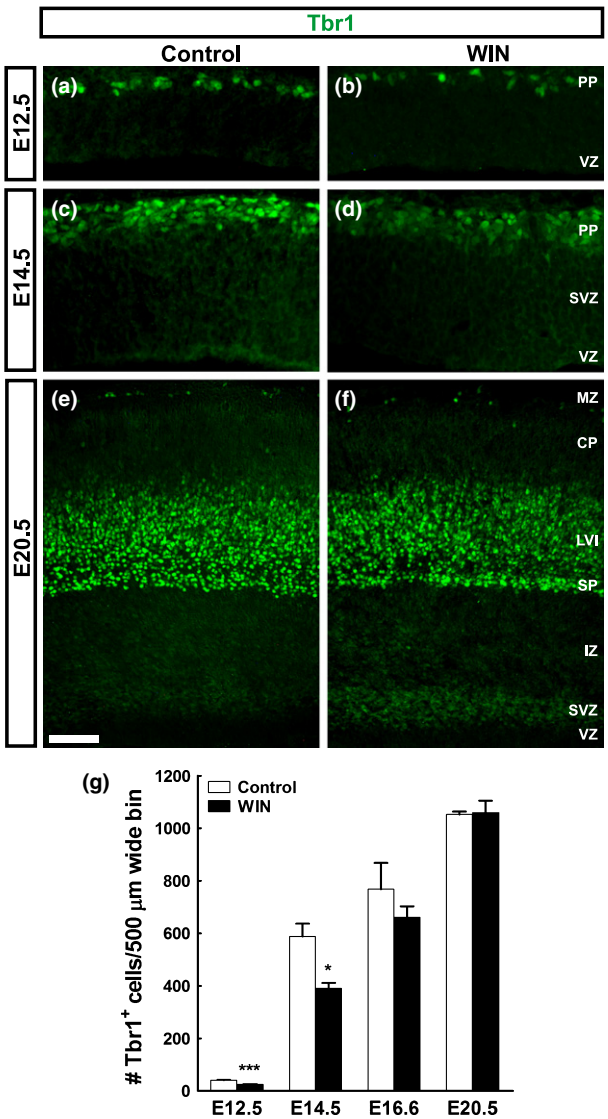

Fig. 6 The number of glutamatergic post-mitotic neurons was decreased in the developing cerebral cortex of WIN-exposure embryos at E12.5 and E14.5. (a–h) dorsal pallium coronal sections immunostained for Tbr1 in control (a, c, e) and WIN-exposed (b, d, f) embryos at E12.5, E14.5, and E20.5. (g) Bars graph show quantification of Tbr1+ cells in the dorsal pallium of control and WIN-exposed embryos. (*$p < 0.05$, ***$p < 0.0005$, Student's *t*-test, error bars represent SEM, $n = 4$ for control and WIN). Scale bar: 100 μm in (a–f).

Spatial and temporal expression of CB_1R in post-mitotic migrating neurons during corticogenesis

Previous studies described CB_1R expression in the developing rodent cerebral cortex in neural precursors, in early stages of neuronal differentiation and, later, in developing axons (Berghuis *et al.* 2007; Vitalis *et al.* 2008). This study indentified $_{CB1R}$ expression in post-mitotic migrating pyramidal neurons and interneurons in the rat's developing cerebral cortex During early stage of corticogenesis (E12.5

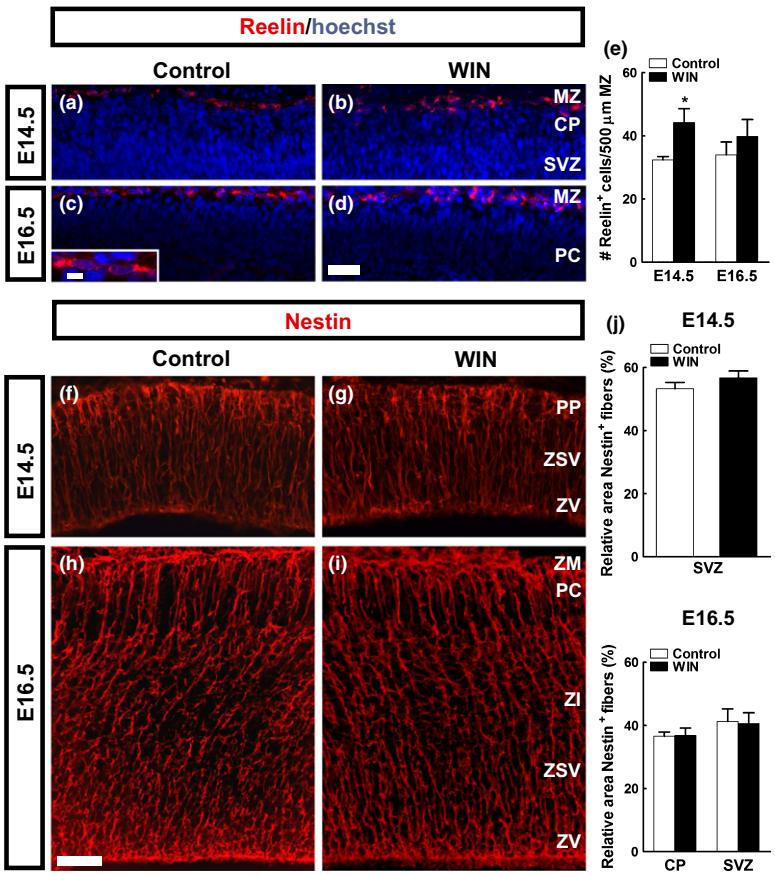

Fig. 7 Effects of prenatal exposure to WIN on Cajal–Retzius cells and radial fibers in the developing cerebral cortex. (a–d) Telencephalic coronal sections immunostained for Reelin from control and WIN-exposed embryos at E14.5 and E16.5. Higher magnification image show a typical horizontal morphology of the Cajal–Retzius cells. (e) Bars graph show quantification of Reelin+ cells in the developing cerebral cortex of the control and WIN-exposed embryos. (*$p < 0.05$, Student's *t*-test, error bars represent SEM, $n = 5$ for control and WIN). (f–i) Telencephalic coronal sections immunostained for Nestin from control and WIN-exposed embryos at E14.5 and E16.5. (j) Bars graph show quantification of relative area of Nestin+ fibers in the developing cerebral cortex of the control and WIN-exposed embryos. (Student's *t*-test, error bars represent SEM, $n = 5$ for control and WIN). Scale bars: 50 μm in a-d, 10 μm in (c) inset, 100 μm in (f–i).

and E13.5), CB1R was expressed in radially oriented migrating neurons at the level of the VZ/SVZ, representing early-born glutamatergic neurons in process of radial migration toward the PP. In addition, we identify CB_1R expression in tangentially migrating interneurons moving into the superficial migratory stream toward their final positions mainly in the hippocampus. In contrast, Berghuis *et al.* reported CB_1R expression in migrating GABAergic interneurons, but only during late stages of corticogenesis as they were undergoing intracortical radial migration (Berghuis *et al.* 2007). On the other hand, Morozov *et al.* identified a subset of CB_1R/CCK+ and CB_1R/reelin/calretinin+ hippocampal interneurons originated in the caudal GE and pallial-subpallial boundary, but they failed to detect co-localization of CB_1R+ cells with interneuronal markers while migrating through the neocortex upon arrival to the hippocampus (Morozov *et al.* 2009). The fact that CB_1R is expressed in migrating post-mitotic neurons since early stages of corticogenesis suggests that eCB signaling is required for the regulation of neuronal radial and tangential migration.

Effects of prenatal exposure to WIN on neuronal migration and differentiation in the developing cerebral cortex

The germinative zones of the dorsal pallium also represent transit regions for migrating neurons. In these layers, we found an increase in the number of radial and tangential post-mitotic migrating neurons in WIN-exposed embryos. Previous studies have shown that acute treatment with HU-210, a synthetic agonist of CB_1R, in organotypic cultures promoted radial migration from the VZ/SVZ to the CP (Mulder *et al.* 2008). Indeed, CB_1R and fatty acid amine hidrolase-deficient mice showed an impaired distribution of cortical pyramidal neurons. In agreement with these findings, we showed that prenatal cannabinoid exposure can impair tangential and radial migration of early-born post-mitotic neurons in the developing rat cerebral cortex. Prenatal exposure to WIN also induced an increase in the number of GABAergic interneurons in the superficial migratory stream. Although we observed an increase in the number of DCX+ cells in the VZ/SVZ, we did not find significant effects of prenatal WIN exposure on the number of GABA+ cells in the deep migratory stream. This could be because of the fact that immature interneurons only acquire GABA expression in the course of their maturation and probably a proportion of tangentially orientated DCX+ cell in the SVZ/IZ, still do not express GABA. Since GABAergic interneurons invade the hippocampus mainly throughout a superficial migratory stream adjacent to the MZ (Manent *et al.* 2006), it would be expected to find a defect in the positioning of hippocampal interneurons in post-natal brain of WIN-exposed offsprings.

This hypothesis is supported by our present finding of the expression of CB_1R in migrating DCX+ post-mitotic neurons tangentially orientated in the superficial pathway. Indeed, prenatal Δ^9-tetrahidrocannabinol-exposure has been demonstrated to induce an increase in CCK-expressing interneurons in the early post-natal rat hippocampus (Berghuis *et al.* 2005). Berghuis *et al.* also showed that *in vitro* pharmacological activation of CB_1R on CCK-interneurons induced chemotaxis through a mechanism involving transactivation of brain-derived neurotrophic factor (BDNF) receptor TrkB. Moreover, it was shown that prenatal WIN-exposure reduced BDNF levels in hippocampus and frontal cortex of the adult offspring (Maj *et al.* 2007). Therefore, over-activation of CB_1R on developing migrating neurons may interfere in the migratory machinery of a subpopulation of interneurons through CB_1R and affect their post-natal positioning and prevent the proper patterning of cortical neuronal networks. Previous evidence suggested that prenatal WIN can permanently alter GABA and Glutamate circuits in the prefrontal cortex and hippocampus (Mereu *et al.* 2003; Antonelli *et al.* 2004, 2005). Failure in specification or migration of pyramidal neurons and interneurons could lead to an abnormal distribution into the cerebral cortex and could result in a disturbance of neuronal activity that in turn would be followed by a neurochemical unbalance.

In vitro pharmacological activation of CB_1R enhanced neural progenitor proliferation (Mulder *et al.* 2008; Trazzi *et al.* 2010); however, we did not find significant effects of prenatal cannabinoid exposure on cell proliferation in the embryonic dorsal pallium. This discrepancy could be because of differences in the experimental model, drug administration and/or the duration of the treatment. Nonetheless, the effect of cannabinoids on neural proliferation has not been unambiguously demonstrated *in vivo*. Therefore, the increase in the number of DCX+ cells in the VZ/SVZ is not because of an increase in cellular proliferation, which in turn reinforces the hypothesis that prenatal WIN exposure interferes with normal neuronal migration.

Although it has been described that chronic exposure to WIN down-regulates pro-apoptotic molecules and up-regulates non-apoptotic ones in the cerebral cortex of adult rodents (Alvaro-Bartolome *et al.* 2010), we did not observe any statistical difference in the number of apoptotic cells in the developing cerebral cortex of WIN-exposed embryos. Since cell death by apoptosis mainly occurs at early post-natal ages, during synaptogenis of post-mitotic neurons, to eliminate neurons which failed to position correctly, it would be expected that any aberrant modulation of apoptotic pathways would be evident at this early ages.

Since Tbr1 is a transcription factor that promotes cortico-thalamic projection neuron specification (Hevner *et al.* 2001) and previous studies have shown that CB_1R inactivation impaired corticothalamic connectivity (Mulder *et al.* 2008; Wu *et al.* 2010), we assessed if prenatal WIN exposure had any effect on this neuronal subpopulation. Here, we showed that WIN treatment delayed the differentiation of intermediate progenitor cell (Tbr2+) to post-mitotic glutamatergic neurons of the deeper layer (Tbr1+), as shown by de increase in the number of Tbr2+ cells and the transient decreased of post-mitotic Tbr1+ neurons. In line with these results, a recent study revealed that CB_1R is able to modulate transcription factors that control pyramidal neurogenesis and upper and lower cortical neuron differentiation. CB_1R-deficient mice showed a delayed distribution of post-mitotic Tbr1+ neurons and fate decision changes through the Ctip2 (COUP-TF interacting protein 2)/Stab 2 (special AT-rich binding protein 2) transcriptional regulation code (Diaz-Alonso *et al.* 2012). With regards to the increase in DCX+ radial post-mitotic neurons in the SVZ, we could speculate that a delay in glutamatergic neuron differentiation could induce a delay in the onset of migration to the CP. Moreover, prenatal WIN-exposure induced a transient increased in Cajal–Retzius cells population at early stage of corticogenesis. Therefore, an impaired in Reelin expression could be a factor of deficit in radial migration. On the other hand, another recent work demonstrated that Tbr2+ cortical intermediate progenitors dictate the migratory route and control the amount of GABAergic interneurons in the SVZ (Sessa *et al.* 2010). Therefore, we could hypothesize that the increase in Tbr2+ cells in WIN-treated embryos could account for the increase in DCX+ tangential neurons in the SVZ.

In conclusion, this study provides a detailed analysis of the consequences of prenatal cannabinoid exposure in the embryonic cerebral cortex. Alteration in the proper regulation of neuronal specification, differentiation and migration of glutamatergic and GABAergic cortical neurons during cortical development might result in an inappropriate assembly of neuronal networks that could in turn lead to a cognitive and neurobehavioral dysfunction later in adult life. In this context, it is important to note that prenatal WIN-induced alteration in cortical glutamatergic and GABAergic neurons are associated with a cognitive and neurobehavioral deficit in post-natal rats (Mereu *et al.* 2003). The present results contribute to the knowledge on neurobiological substrates that determine neurobehavioral changes that will persist through post-natal life.

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