MARK2/Par-1 guides the directionality of neuroblasts migrating to the olfactory bulb

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In rodents and most other mammals studied, neuronal precursors generated in the subventricular zone (SVZ) migrate to the adult olfactory bulb (OB) to differentiate into interneurons called granule and periglomerular cells. How the newborn cells navigate in the postnatal forebrain to reach precisely their target area is largely unknown. However, it is often thought that postnatal neurogenesis recapitulates the neuronal development occurring during embryogenesis.

During brain development, intracellular kinases are key elements for controlling cell polarization as well as the coupling between polarization and cellular movement. We show here that the polarity kinase MARK2 maintains its expression in the postnatal SVZ-OB system. We therefore investigated the potential role of this kinase in adjusting postnatal neuroblast migration. We employed mouse brain slices maintained in culture, in combination with lentiviral vector injections designed to label neuronal precursors with GFP and to diminish the expression of MARK2. Time-lapse video microscopy was used to monitor neuroblast migration in the postnatal forebrain from SVZ precursors to cells populating the OB.

We found that reduced MARK2 expression resulted in altered migratory patterns and stalled neuroblasts in the rostral migratory stream (RMS). In agreement with the observed migratory defects, we report a diminution of the proportion of cells reaching the OB layers. Our study reveals the involvement of MARK2 in the maintenance of the migratory direction in postnatally-generated neuroblasts and consequently on the control of the number of newly-generated neurons reaching and integrating the appropriate target circuits.

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Introduction

Neuronal migration is a key function for proper brain formation since most neurons are born in areas distinct from the place where they will operate. To reach their final destination, neurons employ different migratory modes (Kriegstein and Noctor, 2004; Métin et al., 2008). In the cerebral cortex, excitatory neurons born in the germinal zones of the dorsal telencephalon migrate radially to the pial surface (Hatten, 2002; Kriegstein and Noctor, 2004; Ayala et al., 2007), whilst cortical interneurons migrate rather tangentially across the plane of the glial fiber system (Métin et al., 2008; Marín and Rubenstein, 2001). In embryos, cortical tangential migration is still ongoing when most of the radial migration is accomplished. In contrast, in the OB, postnatally-generated interneurons follow a tangential migration in the RMS before shifting to a radial migration mode to reach superficial cellular layers (Alvarez-Buylla and Garcia-Verdugo, 2002). The tangential migration of neural precursors generated in the SVZ and heading to the OB is maintained throughout life (Altman, 1969; Luskin, 1993; Lois and Alvarez-Buylla, 1994; Lois et al., 1996; reviewed in Lledo et al., 2006).

Neuronal migration is a directional process, which according to the most widely accepted model, depends on the integration of external factors and cell-autonomous processes that determine the cell polarity, dictating the direction of cell movements. Extensive investigation has identified external soluble factors, which repel or attract the neuroblasts in precise directions, according to chemical gradients (Wu et al., 1999; Sawamoto et al., 2006; Murase et al., 2008) and/or cell contact-mediating molecules. These factors include polysialated neural cell adhesion molecule, ephrin-B, members of the Slit and integrin families, prokineticin 2 receptors, and ambient GABA (reviews Lledo et al., 2006; Pathania et al., 2010), involved in the control of tangential migration. Meanwhile, reelin and tenasin-R are identified candidate molecules regulating radial migration (Hack et al., 2002; Saghatelyan et al., 2004).

Although some of the molecular signals involved in the emigration of neuroblasts from the SVZ have been identified, the way neuronal...
precursors find their precise path to reach neuronal circuits in the OB remains poorly understood. In the present study, we questioned whether MARK2, a key player in the polarity pathway (Biernat et al., 2002; Macara, 2004; Pelletier and Seydoux, 2002), participates in neuronal polarization of migrating neuroblasts in the postnatal forebrain. MARK2 is part of the calcium-calmodulin dependent kinase (CaMK) family and is regulated by cAMP, thus representing a good sensor for metabolic as well as neural signaling processes potentially responsible for regulating the neuronal precursors’ migration. MARK2 phosphorylates several microtubule associated proteins such as tau, MAP2/4 and doublecortin (reviewed in Reiner and Sapir, 2009), and this suggested its possible involvement in cellular polarity as well as regulation of migration, previously corroborated in the cortex (Sapir et al., 2008a, b; Reiner and Sapir, 2009).

Our results show that RMS neuroblasts express MARK2 in the postnatal and adult brain. We thus reduced the expression levels of MARK2, using a gene silencing approach based on shRNA interference. This manipulation revealed that MARK2 was important for the correct positioning of the neuroblasts’ leading processes heading toward the OB. Thus, we investigated whether MARK2 could also participate in the migration of postnatally-generated neuroblasts in the SVZ. Decreasing the expression levels of MARK2 in neuroblasts impaired the ability of the cells to maintain a sustained forward direction of displacement in the RMS. As a consequence, the integration of newborn neurons into the OB circuit was compromised. For the first time, this study specifies the function of MARK2 in the migration of RMS neuroblasts during the postnatal period.

**Results**

**MARK2 is expressed in the SVZ-RMS system**

To first document a potential role of MARK2 on postnatal neurogenesis, experiments were performed to reveal whether the kinase is expressed in the neurogenic areas of the postnatal forebrain. We used double immunohistochemistry to characterize the distribution and expression of MARK2 in P8–9 mice. Our results show an abundant expression of MARK2 protein in the SVZ (Fig. 1A, B) and distributed along the RMS in cells that were also positive for doublecortin (DCX) antibody, a selective marker of cells committed to the neuronal lineage in both the developing and the adult brain (Fig. 1C–E). We confirmed that later in development the expression of MARK2 in the RMS was conserved, since we observed numerous cells stained for MARK2 which also express DCX in P60 mice (Fig. 1F–I).

**MARK2 shapes the migration of RMS neuroblasts**

To characterize the role of MARK2 in specifying the migration of postnatal-generated neuroblasts, we performed time-lapse video microscopy on a previously characterized in vitro organotypic culture slice preparation containing the hallmarks of the postnatal forebrain, including the SVZ, RMS and OB (Mejia-Gervacio et al., 2011). Neural progenitors were transduced with lentiviral vectors injected into the SVZ, encoding GFP and shRNA sequence to knock down the expression of MARK2 (Sapir et al., 2008a), or GFP and a scrambled shRNA sequence for controls. Visualizing fluorescent cells in the RMS allowed us to evaluate the speed and the directionality of the migratory displacement of GFP+ cells (Figs. 2 and 3). The average speed of motility was not different between cells transduced with shMARK2 or the control sequence (mean values of 40.08 ± 5.04 μm/h, n = 42 cells from 5 slices and 34.40 ± 4.91 μm/h, n = 67 cells from 5 slices, respectively, p = 0.49). Fig. 2A displays the cumulative frequency of average speed showing no difference between the two groups of cells (p = 0.37 with a Kolmogorov–Smirnov test). The lack of differences between distributions is confirmed by the linear relation between the first and third quartiles of both distributions (Fig. 2A, inset). However, a more detailed analysis of the distribution of speed during the individual 20 min steps of the time-lapse recordings indicates a distinct distribution of fast and slow migratory steps in each cell group. The shMARK2 group showed both an increase in the number of very slow movements and in the frequency of the fastest displacements, per time frame (Fig. 2B). Notice that a stable plateau accounting for 98% of the population values was reached by control cells at 3.6 μm/s meanwhile the plateau was observed at 5.8 μm/s in shMARK2 cells. The increase in the frequency of very slow and very fast movements at the expense of the intermediary migration speeds in the shMARK2 group accounts for the lack of differences in the average speed. The differences between the groups distribution became significant when considering only the very slow movements (mean values of 0.34 ± 0.24 μm/h, n = 42 cells from 5 slices, respectively, p = 0.006).
In control, the majority of GFP+ cells had their leading process pointing towards the OB when progressing forward (Fig. 3A). We analyzed the orientation of 42 cells from 5 slices, the shMARK2 expressing cells showed a net tendency to move forward, i.e. towards the OB, with mean values of 14.88±6.70 μm/hr. Backward movement was also observed in control cells, but a conspicuous difference was noted when examining the total displacement of the cells during the entire session. Whereas control cells showed a net tendency to move forward, i.e. towards the OB, shMARK2 RMS neuroblasts, with a leading process oriented towards the OB. A close examination of the time-lapse movies indicated a striking abnormality in the directionality of the shMARK2-treated cells. A large proportion of these cells showed fast backwards displacement, towards the SVZ (Fig. 3C). Backward movement was also observed in control cells, but a conspicuous difference was noted when examining the total displacement of the cells during the entire session. Whereas control cells showed a net tendency to move forward, i.e. towards the OB, with mean values of 14.88±6.70 μm/hr with respect to their location at the beginning of the session (n=42 cells from 5 slices; Fig. 3C). Accordingly, the statistical analysis shows that the total average displacement of the control group, but not the shMARK2 group was significantly different from zero (Fig. 3C, p=0.04 and p=0.14, respectively). Together, these results show that despite the fact that shMARK2-expressing cells are capable of motility with normal mean speed (see above), the appropriate migratory directionality was impaired compared to control cells.

**MARK2 does not determine the neuroblasts’ morphology**

To check for a possible alteration in the cellular morphology following reduction of the MARK2 expression level, we analyzed the pattern of migration is modiﬁed in cells with decreased MARK2 levels. A) Plot showing the cumulative frequency of average speed of migration during the whole recording session in cells expressing the control, non-target sequence (gray), as well as in cells expressing the shMARK2 sequence (blue). Inset. Quantile–Quantile plot showing no differences in the distribution of average speed calculated for control and shMARK2 cells during the entire recording session. The dashed line represents a linear fit between the first and third quantiles, shown as deep blue markers. B) Plot of the cumulative frequency of migration speed on 20 min individual steps in control and shMARK2 expressing cells. Double arrow indicates the increased frequency of slow movements observed in shMARK2 expressing cells. Arrows show the value corresponding to 98% of the population observations for control (gray) and shMARK2 groups (blue). Inset. Quantile–Quantile plot of migration speed for individual 20 min steps showing the deviation from a linear relationship between the distributions. Explanations as in A. The same cells were analyzed in A and B. ** p < 0.01.

### Fig. 2.

The pattern of migration is modiﬁed in cells with decreased MARK2 levels. A) Plot showing the cumulative frequency of average speed of migration during the whole recording session in cells expressing the control, non-target sequence (gray), as well as in cells expressing the shMARK2 sequence (blue). Inset. Quantile–Quantile plot showing no differences in the distribution of average speed calculated for control and shMARK2 cells during the entire recording session. The dashed line represents a linear fit between the first and third quantiles, shown as deep blue markers. B) Plot of the cumulative frequency of migration speed on 20 min individual steps in control and shMARK2 expressing cells. Double arrow indicates the increased frequency of slow movements observed in shMARK2 expressing cells. Arrows show the value corresponding to 98% of the population observations for control (gray) and shMARK2 groups (blue). Inset. Quantile–Quantile plot of migration speed for individual 20 min steps showing the deviation from a linear relationship between the distributions. Explanations as in A. The same cells were analyzed in A and B. ** p < 0.01.

### Fig. 3.

The directionality of the migration is affected by the knock down of MARK2 expression. A) Photomicrograph showing GFP+ migrating neuroblasts in the RMS. The neuroblasts make back or forth displacements in the RMS following the direction of the leading process (blue arrow heads). Calibration bar 20 μm. B) Percentage of control and shMARK2 RMS neuroblasts, with a leading process oriented towards the OB. C) Analysis of the migration directionality during the experimental session in control and shMARK2 expressing cells. Cellular displacements towards the SVZ hold negative values and movements towards the OB are positive. Each point represents the total distance covered by one cell during the entire experimental session and the cells’ initial position is at the origin. Average ±SEM values for each group are shown in green. ** p < 0.01.
The integration and survival of neuronal precursors are sensitive to MARK2

The precursors for neurons in the RMS give rise to interneurons that will integrate in the granule cell (GC) and glomerular layers of the OB. Using a method of analysis that allows the recognition of equivalent regions of interest in the OB (Suppl. Fig. 1), we quantified the number of GC integrating the GC layer (GCL) and periglomerular cells (PG) integrating the glomerular layer (PGL) in the OB, using slices fixed after 12 DIV (Fig. 5). During the first few days of culture, the number of RMS cells was equivalent in both groups. Nonetheless, after 12 DIV, the number of cells integrating both the GCL and GL was significantly lower in the shMARK2 group compared to the control. The cell counting revealed a difference in the average number of cells integrating the GCL of 2.2 ± 0.3 cells versus 1.1 ± 0.3 cells for control and MARK2 shRNA slices respectively (p = 0.01). Meanwhile, average values of 6.3 ± 1.6 versus 3.3 ± 0.4 cells were counted in the PGL (p = 0.001; Fig. 6A). The observed reduction in the number of cells was not accompanied by morphological changes of the GCs, as evaluated by sholl analysis in slices fixed at 12 DIV (n = 6 and 5 cells; see Suppl. Fig. 2).

To determine whether the diminution in the number of cells integrating the appropriate layers in the OB resulted from an accumulation of cells in the RMS, we quantified the number of cells in equivalent regions of interest in the RMS at 12 DIV. The number of cells was, once again, significantly lower in the shMARK2 group compared to the control group (0.72 ± 0.16 and 2.21 ± 0.66 cells; p = 0.04, respectively).

Thus the defects of integration of newly generated interneurons to the OB does not result from long-lasting accumulation of precursors in the RMS. Similar results were obtained when data were expressed as cell density rather than cell number (Fig. 5B). In the three regions analyzed, a reduction was found in the shMARK2 group compared with the control. The cell density values were 5.18 × 10⁻⁶ ± 1.09 × 10⁻⁶ cells/µm² in control and dropped to 1.92 × 10⁻⁶ ± 5.57 × 10⁻⁶ cells/µm² in the shMARK2-expressing group (p = 0.004), in the RMS. Similarly, in the GCL values diminished from 5.33 × 10⁻⁵ ± 0.90 × 10⁻⁶ cells/µm² in control to 2.78 × 10⁻⁵ ± 4.82 × 10⁻⁶ cells/µm² in shMARK2 cells (p = 0.01). In the GL, a non-significant reduction was also observed from 6.43 × 10⁻⁶ ± 7.43 × 10⁻⁶ cells/µm² in control to 5.33 × 10⁻⁶ ± 9.09 × 10⁻⁶ cells/µm² in the shMARK2 group (p = 0.77; Fig. 5B).

Altogether our results suggest that MARK2 plays a role in the control of the migration patterns and direction of motility in RMS neuroblasts and thus ensure the appropriate arrival of newborn neurons to the OB. Accordingly, reduced levels of MARK2 resulted in a reduced number of newly-generated neurons in the OB.

Discussion

The arrival of neuroblasts to the OB occurs throughout life and provides the OB with a continuous supply of new GCs and PGs (reviewed in Marín and Rubenstein, 2001; Lidér et al., 2006). The tangential migration of neuroblasts in the RMS is controlled by a series of repellent cues, which are intensely studied (Wu et al., 1999; Kaneko et al., 2010; Sawamoto et al., 2006; Nguyen-Ba-Charvet et al., 2004; Mason et al., 2001). However, the molecular effectors determining the appropriate polarization of the cells to follow the external cues is largely unknown. In previous studies, we have shown that members of the polarity pathway, notably MARK2 protein determine the polarization of the excitatory interneurons of the cortex, in a way compatible...
with their appropriate migration (Sapir et al., 2008a, b) Since the same molecular effectors are present in different developing regions of the postnatal brain and frequently associated to similar functions, we decided to explore whether the migration of RMS neuroblasts was sensitive to the activity of MARK2.

Our results show that MARK2 is expressed in the postnatal and adult RMS, and according to our time-lapse videomicroscopy experiments, this protein is functional at early stages of development in RMS neuroblasts. In agreement with its role on cellular polarization, our results suggest that in the RMS, MARK2 regulates the polarization of the leading process of migratory neuroblasts; and its disruption significantly affects the directionality of the neuroblasts’ migratory displacement. Specifically, the knockdown of MARK2 in the RMS produced an impaired migration, characterized by increased futile back and forth displacement resulting in no net migration towards the OB. Since in our assay the neuroblast migration is evaluated several micrometers away from the injection site of shRNA MARK2 (see Mejía-Gervacio et al., 2011), we believe that the observed effects are due to cell-autonomous and not to niche effects in the SVZ.

The observed defects on the migration associated to the dysfunction of MARK2 in the RMS were expected from the previous results in radially migrating neurons in the cerebral cortex. The characteristics of the defects on migration and consequently the role of MARK2 in these two developing regions differ in several aspects discussed below. These differences highlight the specific contribution of these molecular effectors according to the respective location of the neural niches.

In the cortex, the reduction of MARK2 expression resulted in stalled neurons with multipolar structure (Sapir et al., 2008a). The study in the cortex focused on radially migrating neurons, meanwhile the present study addresses the role of MARK2 on the tangential migration in the RMS. Accordingly, differences were observed when comparing the characteristics of the migration defects in these two areas. First, in the RMS, the altered migration was not associated to changes in the shape or characteristics of the migration defects in these two areas. First, in the RMS, the altered migration was not associated to changes in the shape or length of the neural projections (see Fig. 4). Moreover, the defects on the directionality in shRNA MARK2 cells in the RMS were not accompanied by a general drop of the speed of migration but rather by a change in the pattern of migration, leading to a displacement characterized by long pauses but less frequent and larger displacement, in contrast to the more regular migratory steps observed in the control neuroblasts.

In the cortex, the polarization of the centrosome leads the nuclei displacement and controls neuroblast migration (Valiente and Marín, 2010). In the cortical neuroblasts, MARK2 significantly reduced the velocity of centrosome movement, resulting in stalled neurons (Sapir et al., 2008a, b). In the RMS, although the centrosome location does predict the nuclei positioning after nucleokinesis, the direction trend of the leading process or the nuclei displacement are not determined by the centrosome migration (Schaar and McConnell, 2005). Thus it is unlikely that our altered migration is associated with a lack of polarization of the centrosome. Instead our results point to the role of MARK2 in determining the stable polarization of the leading process towards the direction of the target region.

From the molecular point of view equivalent differences concerning the migration in the cortex and the RMS can be readily identified. For example, in radially migrating neurons the reelin pathway affects neuronal migration through the receptor proteins ApoER2 and VLDLR transmitting the signal downstream to Dab1 (review Tissir and Goffinet, 2003). Reelin is not present in the RMS, however the absence of either the receptor or Dab1, in knockout mice, impaired the formation of chains of migrating neuroblasts and resulted in an accumulation of neuroblasts in the SVZ (Andrade et al., 2007).

MARK2 has several molecular partners operating up- and downstream in metabolic pathways associated to different processes of neuronal development such as axon and dendrite formation and migration (Reiner and Sapir, 2009). MARK2 is phosphorylated by the activity of CaMK1 and other members of the Par family as well as by the atypical PKC pathway (reviewed in Marx et al., 2010). On its side, MARK2 phosphorylates several microtubule associated proteins such as tau, MAP2/4, DCX and kinesin-like motor proteins (see Reiner and Sapir, 2009; Yoshimura et al., 2010).

It is very likely that in the RMS, MARK2 acts via its association with cytoskeletal associated substrates, as in the cortex. To this respect DCX expression is particularly abundant in the RMS (Timm et al., 2006; Sapir et al., 2008b; Belvindrah et al., 2011). In a similar way, shared molecular effectors such as the kinase Cdk5, important for the regulation during radial neuronal migration, might interact with MARK2 to regulate the tangential migration of the RMS (Dhawan and Tsai, 2001; Gupta et al., 2002; Ayala et al., 2007). Accordingly, it has been previously shown that Cdk5 deletion impaired the chain formation, speed, directionality, and leading process extension of the SVZ neuroblasts in a cell-autonomous manner (Hiroti et al., 2007). In this respect, it is relevant that Cdk5 and MARK2 have multiple common substrates associated with the cytoskeleton (Smith, 2003).

The most relevant consequence of the defects of migration concerns the poor integration of new interneurons in the OB. Our results show that this aspect is also affected in shMARK2 expressing cells. A diminished number of cells was observed in the OB layers but also in the RMS after 12 DIV, indicating no accumulation of the migration-impaired cells in the RMS. These results might indicate the elimination of cells impaired for migration after some days in the RMS.

Collectively, our results suggest that MARK2 plays an essential role in the polarization of the neuroblasts’ leading process in the RMS and thus determines the direction of the migration to the OB. Together with previous reports in the cerebral cortex our results support the importance of the polarity pathway elements on the neuronal migration. The control of the directionality of cellular displacement is of great importance not only because it ensures the correct targeting of newborn cells during development but also has very obvious consequences for the pathology after brain lesions. In the RMS, several studies have shown evidence of the re-routing of neuroblasts to injured areas in the brain after different kinds of lesions (Arvidsson et al., 2002; Sundholm-Peters et al., 2005). Further studies should address whether MARK2 also plays a role in re-routing the migration of the new cells to populate injured brain regions.

Experimental methods

Culture preparation

Sagittal slices from postnatal day 7 (P7) C57BL/6J mouse pups (Janvier) were cultured and cells in the SVZ were transduced with shRNA sequences, introduced using lentiviral vectors, as previously described (Mejía-Gervacio et al., 2011). Briefly, mice were decapitated and brains were removed into ice-cold ACSF containing (in mM): 124 NaCl, 3 KCl, 1.3 MgSO4, 26 NaHCO3, 1.25 NaH2PO4, 10 Glucose and 2 CaCl2, saturated with 95% O2/5% CO2. Sagittal forebrain slices, 300 μm thick, containing the migratory pathway from the SVZ-RMS and OB were obtained using a vibratome (Leica VT1200). All experimental procedures were performed in accordance with the Charter of Fundamental Rights of the European Union (2000/C 364/01), the European Communities Council Directive of 24 November 1986 (86/609/EEC), and European Union guidelines, and were reviewed and approved by our Institutional Animal Welfare Committee.

Following the method by Stoppini et al. (1991), each brain slice was transferred to a Millicell-CM Culture Plate Insert (Millipore PiCM ORG 50), the excess ACSF was removed and the insert was placed in a 35 mm Petri dish, containing 1 ml of medium. Culture medium consisted of 46% MEM (31095-029), 25% HBSS (24020-091), 25% horse serum, 20 mM HEPES, 6 mg/ml NG-glucose and Gentamycin (Invitrogen). The slices were incubated at 37 °C in a humid atmosphere of 5% CO2. Media was changed three times per week.

Lentiviral vectors

We used a lentivirus with an inserted Lentilox 3.7 vector encoding for GFP under the control of the CMV promoter and either a short
hairpin RNA (shRNA) sequence for MARK2 (5′-GAGGATGCTGTGAA-GATCA3′) or a non-target scrambled sequence (5′-TAAGCTATGGA-GAGTAC3′) (Cohen et al., 2008; Sapir et al., 2008a) under control of the U6 promoter. The efficacy of the shRNA MARK2 sequence as well as the scramble sequence was corroborated in a previous study using real type PCR and immunohistochemistry (Sapir et al., 2008a).

One of the viral constructions was injected with an automatic injector (NanojectII, Drummond) 24 h after culturing the slices (90 pg of p24 in a volume of 18 nl) in the SVZ to induce the corresponding sequence expression in neural precursors and neuroblasts in the SVZ.

Time-lapse video microscopy

The Petri dish containing the cultured slice was transferred to a closed chamber with temperature control adjusted to keep the medium at 36 °C (DH40 Warner Instruments). During the entire time-lapse session, the tissue was oxygenated using a mixture of 95% O2/5% CO2. To prevent evaporation of the culture medium, the internal environment of the chamber was continuously humidified (Humidifying module warm HWM-1, Warner Inst). Mosaic images of the entire RMS were re-constructed from individual frames (X-, Y- and Z-axis), acquired each 20 min during 3 h. We used an inverted fluorescence microscope (Axioscope FS, Zeiss) equipped with a 25× objective (LD plan Neo, Zeiss). For analysis, a maximal projection of the different planes taken in the Z-axis was obtained for each lapse of time. Individual RMS neuroblasts could be identified and their trajectories tracked using Metamorph (Molecular devices). Two parameters of the migration were analyzed: the speed and the displacement in the X-axis, since the rostro-caudal axis of the RMS was accommodated to be parallel to the X-axis. Delta X (ΔX), was used as an index of the direction of the migration of the cells in the RMS-OB path.

Analysis of cells integrating the OB

To test whether the cells with impaired MARK2 expression do integrate or not the OB network, the slices were fixed after 8 or 12 days in vitro and high-resolution images containing the entire path from the RMS to the OB were acquired (Suppl. Fig. 1). Three to four optical planes (6 μm) were acquired from each slice using an inverted fluorescence microscope (Axioscope FS, Zeiss) equipped with a 25× objective (Zeiss) and the global mosaic image was reconstructed. We compared the number of GFP+ cells in the RMS, granule cell layer (GCL) and glo- merular layer (GL) of slices expressing either the control or shMARK2 sequences. Since the morphology of the GCL and PGL changes depending on the lateral plane under study, a specific anatomical pattern, corresponding to the dimensions of these layers in each lateral plane was superimposed over the slices. This method allows the recognition of equivalent regions of interest in the RMS, GCL and PGL that could be compared between slices (Suppl. Fig. 1). For quantification, three regions were identified in the RMS and four to five different regions were identified in the GCL (depending on the lateral plane of the slice). We manually quantified the total number of GFP+ cells contained in each of these regions. The limits of the GL were also determined and the entire population of GFP+ periglomerular cells was quantified. The results were expressed both in density of cells per μm² as well as the total number of GFP+ cells quantified.

Leading process polarization and morphological analysis

Brain slices were fixed in 4% paraformaldehyde, before being cryoprotected in 12.5% sucrose/5% glycerol 2 h at 4 °C followed by an overnight incubation in 25% sucrose and 10% glycerol. Slices were rapidly frozen and thawed three times over liquid N2 vapor, washed in PBS then kept at RT 2 h in 10% FBS 0.1% Triton blocking solution. GFP labeling was amplified using an Upstate antibody (06–896) at 1:1000 in blocking solution for an incubation of 48 h at 4 °C followed by washes in PBS. Slices were incubated for 2 h in Alexa-conjugated secondary antibody (1:750, Molecular Probes) at RT, washed in PBS then mounted in ProLong Gold antifade (Molecular Probes P36930). Images were acquired using a 25× objective mounted in an Axio- scope Observer Z1 equipped with an Apotome (Zeiss).

The direction of the polarization of the leading process towards the OB or towards the SVZ was quantified as total number of cells in the RMS with a leading process oriented in any of these directions in 8 DIV slices. To evaluate potential morphological differences between cells expressing control or shMARK2 sequences, we used the Sholl index to compare the number of intersections crossing a radial circumference initiating from the cell body. The intersections corresponded to the leading and trailing processes in RMS neuroblasts and to dendritic projections in the GC. For the analysis we used 5 μm radial steps for the neuroblasts and 10 μm radial steps for the GC.

Immunohistochemistry

P8-P9 or adult mice were fixed by intracardiac perfusion of 4% paraformaldehyde before brain dissection. Brains were postfixed overnight in 4% PFA-PBS immersed in 20% sucrose for cryoprotection and cryosectioned. Alternatively, following fixation slices were obtained with a Vibratome (Leica VT1000S). Floating sections were permeabilized in PBS 1%, FBS (Invitrogen) 0.2%, Triton X100 1 h prior to an incubation (48 h/4 °C) with doublecortin polyclonal goat antibody (DXC Santa Cruz, SC-8066) and Rabbit anti MARK2 (SA5985 or SA4633; both kindly provided by E-M Mandelkow lab). Slices were incubated for 2 h in Alexa-conjugated secondary antibodies (1:750, Molecular Probes) at RT, washed in PBS. Cell nuclei were stained with DAPI. Slices were mounted in ProLong Gold antifade (Molecular Probes P36930). Images were acquired using a 25× objective mounted in an Axioskope Observer Z1 equipped with an Apotome (Zeiss).

Statistical analysis

Results are expressed as average ± SEM. The comparisons between the control and experimental groups were analyzed using the Student t-test. In other cases ANOVA comparison was used with Newman–Keuls as post-hoc test. Comparisons of distributions were performed using the Kolmogorov–Smirnov test in R program (www.r-project.org). Differences were considered significant at p<0.05.

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References


