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Ex vivo imaging of postnatal cerebellar granule cell migration using confocal macroscopy

--Manuscript Draft--

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Abstract:	During postnatal development, immature granule cells (excitatory interneurons) exhibit tangential migration in the external granular layer, and then radial migration in the molecular layer and the Purkinje cell layer to reach the internal granular layer of the cerebellar cortex. Default in migratory processes induces either cell death or misplacement of the neurons, leading to deficits in diverse cerebellar functions. Centripetal granule cell migration involves several mechanisms, such as chemotaxis and extracellular matrix degradation, to guide the cells towards their final position, but the factors that regulate cell migration in each cortical layer are only partially known. In our method, acute cerebellar slices are prepared from P10 rats, granule cells are labeled with a fluorescent cytoplasmic marker and tissues are cultured on membrane inserts from 4 to 10 h before starting real-time monitoring of cell migration by confocal macroscopy at 37 °C in the presence of CO2. During their migration in the different cortical layers of the cerebellum, granule cells can be exposed to neuropeptide agonists or antagonists, protease inhibitors, blockers of intracellular effectors or even toxic substances such as alcohol or methylmercury to investigate their possible role in the regulation of neuronal migration.
Author Comments:	I have been contacted by Jane Hannon in the early summertime of 2014.
Additional Information:	
Question	**Response**
If this article needs to be "in-press" by a	Feb 27, 2015

certain date to satisfy grant requirements, please indicate the date below and explain in your cover letter.	
If this article needs to be filmed by a certain date to due to author/equipment/lab availability, please indicate the date below and explain in your cover letter.	

Mont-Saint-Aignan, December 26th, 2014

To
Nam Nguyen
Science Editor
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1 Alewife Center, Suite 200
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From
Dr Ludovic Galas
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Dear Nam Nguyen,

Attached please find a revised version of our manuscript entitled "*Ex vivo* imaging of postnatal cerebellar granule cell migration using confocal macroscopy" (Ms # JoVE52810).

The detailed review of our manuscript was greatly appreciated, and we have attempted to address all issues. Below, please find our detailed point-by-point responses to reviewers in bold fond. In the revised manuscript, we have highlighted all changes in red.

Point-by-point responses

Reviewer #1:

Remarks
1. The authors state that also other brain areas can be studied with this protocol. Would the method also be applicable for other organs where cell migration plays a role (e.g. the epithelium of skin and intestine)?

The cell migration protocol described in this paper could be applicable to other tissues with adjustment of experimental conditions (composition of culture medium, nature and concentration of probe for cell staining,…) and, if necessary, slices preparation (frequency and speed of sectioning). Since our present expertise is restricted to neuronal migration, we have focused on brain aspects in the discussion section (lines 384 and 385).

2. I miss information about the pH used and the way to keep the pH constant. (Living cells release waste products that may change the pH of the incubation medium, during the 12 hr duration of the observations.)

The stability of pH has been considered in each step of slices preparation and observations. Slices were prepared in Hank's BSS (HBSS) containing NaHCO3 (350 µg/ml) to adjust and stabilize the pH around 7.6 (lines 129-130). During observations, the slices were supplied with constant gas flow (95% O2, 5% CO2) through the plate insert (line 235-236). Under these conditions, the color of phenol red included in the DMEM- F-12 culture medium did not change, showing a constant value of the pH during the 12 hr of the experiments. To allow readers to get easily the information, we added "to maintain the pH constant" line 240.

3. I would like to see evidence that the decline of the speed of migration upon administration of ligands (Fig. 4) is not an ex vivo effect due to deteriorated viability of the cells from 2 hours on. This would ask for showing the cells' behaviour in a separate study in the control situation, not for 2 but for at least 4 hours.
Moreover, statistical analysis of the data in Fig. 4 should confirm that the decline in speed after ligand administration is statistically significant. Have the authors found substances (agonists) that increase migration speed?

We have been contacted by JoVe editors after the publication of JNC 2014 Jul;130(2):241-54. doi: 10.1111/jnc.12714. Epub 2014 Apr 19, to propose a manuscript dedicated to neuronal migration studies through confocal macroscopy. All control situations have been already proposed in this previous manuscript (in figures or supplementary data) or in other manuscripts where cell migration was observed through confocal microscopy (Cameron et al., Neuroscience, 146, 697-712, 2007; Yacubova and Komuro, Nature, 415, 77-81). The comments of the referee #1 on control situation and statistical analysis are fully rightful but have been addressed for the JNC paper and we want to be careful to not duplicate the data and focus on technical aspects that have not been described in detail previously. In addition, we have demonstrated in other papers that Ca2+ (Fahrion et al., PNAS, 109, 5057-5062, 2012) and IGF-1 (Li et al., PNAS, 109, 2630-2635, 2012) increases the speed of migration of GC.

4. Following one particular migrating cell among many other fluorescent cells is not easy, even not in a large field of observation. In the Discussion attention should be paid to the way this is accomplished.

Cell tracking has been clearly described in detail in the Protocol section from line 255 to line 270. To give easy guidelines to readers, we propose to add in the video, a new step related to paragraph 6 entitled "Cell tracking" in which the way to accomplish the following of migrating cell in a cerebellar slice. Therefore, we have identified this new step in yellow from line 255 to line 264.

5. Explain into more detail in the Discussion why the protocol provides better stabilization of the tissue (less tissue movement) than other methods, and how this has been verified.

After sectioning and labeling, slices attached directly and spontaneously to the porous insert membrane and are covered by a thin film of medium allowing gaz exchange and nutriment access. Our protocol avoids any further detachment, any transfer from the membrane to Petri dish, as well as any additional way (nylon mesh or collagen) to stabilize slices (line 339). Consequently, slices stay in a unique environment after preparation and all along observation. The stabilization of the tissue during image acquisition is verified by following the position of slice edges and fixed points inside the tissue including Purkinje cells that have completed their migration at P10.
To answer to the comment of the referee #1, we have added lines 346-347, the following sentence: "Stabilization of the tissue is verified by following the position of slice edges or Purkinje cells that should be fixed references during acquisition.

6. Why was the heavy point of cells determined?

The gravity point of each cell body was determined as a reference position to track objectively GC during their migration. In order to be more accurate in the description of cell tracking, we added line 263, the gravity point of each cell BODY during time-lapse.

Textual suggestions

Line 86: the cells begin to enter into a short vertical descending process into the ML: skip "into"
Line 87, the first into was suppressed.

Line 88: trailing process, a more voluminous leading process and migrate radially -> trailing process and a more voluminous leading process, and migrate radially
Line 89, the text has been modified as recommended.

Line 94: Some regulatory factors of GC movements including neuropeptides have been identified so far: give examples (e.g. PACAP)
Line 96, (e.g. somatostatin, PACAP) has been added.

Line 327: migration including agonist or antagonist of neuropeptides -> migration including agonists or antagonists of neuropeptides
Line 333, the text has been modified as recommended.

Line 341: the plate can be quickly observed in details -> the plate can be quickly observed in detail
Line 348, the text has been modified as recommended.

Line 343: The sentence "Therefore, time-lapse experiment through confocal macroscopy is like direct observation in a CO2 incubator." is not fully clear. Please explain by rephrasing.

To observe tissue slices through confocal macroscopy, you only have to take the culture plate from the incubator and to put it under the objective situated in a large chamber in which environment is similar to the incubator. Consequently, simplicity of the transfer and similar environment let think that observation under confocal macroscopy is similar to a direct observation in a CO2 incubator.
To satisfy the request of referee #1, the sentence line 352 now reads: Therefore, environmental parameters and culture support similarities in CO_2 incubator and confocal macroscopy lead to maximum conservation of the biological sample.

Line 358: Long time of incubation (up to 8 hours) is necessary -> Long incubation times (up to 8 hours) are necessary
Line 369, the text has been modified as recommended.

Reviewer #2:
Manuscript Summary:
In this manuscript, Bénard et al. describe a technique to acutely label and macroscopically image live cerebellar granule cells in postnatal rat cerebellum during their migration process. This technique could be useful when a fast screening approach is required for identifying molecules or signaling pathways involved in neuronal migration. However, the authors only focus on radial migration along the molecular layer. In this reviewer's opinion the impact of the paper would be broader if the authors show that the same technique can indeed be applied to study migration in other brain areas. Still, several major concerns should be addressed.

Major Concerns:
1. A first major concern is whether the cell tracker dye that the authors use labels specifically granule cells. If the authors claim that this technique is specific for studying postnatal cerebellar granule cell migration, it should be clearly demonstrated, shown and stated in the text that this dye will only (or mainly) label cerebellar granule cells. Without the proper validation controls the described technique lacks essential information. How sure are the authors that they are not labeling other internueurons in the molecular layer? And what about granule cells in the IGL, are they labeled also? In Fig.3A, this does not seem to be the case despite the fact that at P10 in rats there are quite a lot of granule cells that have already reached the IGL.

We did not claim that the cell tracker green labels specifically GC but we only focus on this cell type in this paper since the purpose was to describe the originality of confocal macroscopy approach. From line 381 to 383, we have indicated that interneurons can also be labeled as shown by our previous paper JNC 2014 Jul;130(2):241-54. doi: 10.1111/jnc.12714. Epub 2014 Apr 19. In addition, from line 369 to line 370, we have clearly indicated guidelines to follow GC in other cortical layers including the IGL. Please see also our previous papers including Cameron et al., 2005 in which cell tracker green labeled-GC has been followed in all cortical layers of the cerebellum in mice.

2. A second major concern is regarding the quantification of the migration speed that the authors perform in Fig.3C and in Fig. 4A and 4B:
a) A detailed explanation of the number of neurons analyzed for calculating each point of the migration curve is missing. Or is this migration curve just representative for one single neuron? if so, what is the meaning of having a macroscopic approach, where many neurons can be recorded at the same time, and then show only tracks for single neurons?
b) Error bars for each point are missing
c) There is a big inconsistency with the results shown for migration under basal conditions of Fig3C or Fig4B when compared to Fig4A. In Fig3C the average of migration is 18µm/h, in 4B is 19.2 µm/h but in 4A it is 11.9 µm/h. How can it be that there are such big differences between experiments under basal conditions? This issue should be clarified.
d) Finally, when authors apply PACAP and PAI-1 they claim that these compounds inhibit GC migration. However, the number of cells analyzed nor the number of independent experiments performed are indicated. Moreover, error bars and statistics should be performed to clearly demonstrate, and thus state, that PACAP and PAI-1 block GC migration.

Again, the purpose of this paper was not to reproduce the previous work in JNC 2014. Figures 3C,A and 4B are original data, are not present in the JNC paper and represent single cell as described in the legends. Consequently, there is no error bars. Differences in the speed of GC migration in the ML are generally observed (from 10 to 20 µm/h). However, we did not observed differential sensitivity to regulatory factors tested so far. Whether these differences in rate of migration are associated to GC subtypes deserves further investigation. For the all story about the effect of PACAP and PAI-1, please refer to Raoult et al., Journal of Neurochemistry 2014 Jul;130(2):241-54. doi: 10.1111/jnc.12714. Epub 2014 Apr 19.

3. The authors mention that their method could also be adapted to study the migration of the other types of neurons in the cerebellum or cerebrum at different developmental stages. It is thus interesting for scientists in different neurodevelopmental fields that an example of other type of migration is shown. If the authors want to limit to the cerebellum, they could for example, cut the cerebellum in transverse sections and analyze tangential migration of cerebellar granule cells.

Please refer to answer to question 1 for other type of neuron in the cerebellum. We are currently studying cell migration in the cerebrum but it is not the purpose of the study. We have clearly indicated in the text lines 93-94 and lines 367-368 that transverse sections were necessary to study the tangential migration in the cerebellum and did it previously in the paper from Cameron et al., 2005.

4. Rats are used for some experimental approaches, however it is more common to study neuronal migration in mice (i.e. due to the high number of transgenic mouse lines available). As the described technique could also be applied to mouse cerebellar granule cell migration, where migration rate could be compared between wildtype and transgenic or knockout mice, it is

important that the authors also show that it works in mice and if so, also discuss the advantages of using mice versus rats.

We have also performed a number of studies study in mice with the cell tracker green (Cameron et al., 2005). The confocal macroscopy approach is not species-dependent. Historically, the project on PACAP and tPA was performed on rats for laboratory-specific strategies but rats do not offer particular advantages. However, we did not mention in the paper that it was the case.

5. The authors should discuss limitations (i.e. troubleshooting table) and alternative approaches to this technique in the discussion of the manuscript.
Confocal macroscopy and alternative approaches, including sample preparation, cell labeling strategies and limitation (lateral resolution) have been already discussed in the manuscript. In the introduction from line 100 to line 120 and in the discussion section from line 338 to line 354, from line 357 to line 362 and from line 378 to line 381.

Minor Concerns:
1. Authors should check the spelling mistakes throughout the text (i.e. line 83: "modify" instead of "modifify")
Spelling mistakes have been corrected

2. Consistency should be applied when using abbreviations for differentunits or terminology (i.e. sometimes it is mentioned: h or hr, for "hours")
To satisfy request of referee #2, h is now used for hour in the all text.

3. Authors should mention what type of commercial confocal macroscope they use
The type of commercial macroscope (TCS LSI, Leica Microsystems) has been already mentioned in Table 1.

4. To make it more visual and clear, authors should specify in figure legend 1 what cells are represented by the different colors, in addition to the labeling.

As proposed by the referee #2, association of figure color to cell-types has been added in the legend as followed from line 301 to line 304:
GC, granule cell, in red; B, Bergmann glia, in dark purple; G, Golgi cell, in yellow; cf, climbing fibers, in blue; g, postmigratory granule cells, in light green; mft, mossy fiber terminal, in dark green; P, Purkinje cell, in light purple

5. In the abstract the authors mention: "...but the factors that regulate cell migration in each cortical layer are still largely unknown". This sentence should be tune down as there are indeed many well know factors and mechanisms that influence migration of cerebellar granule cells during postnatal development.

As suggested by the referee #2, the sentence in the abstract (line 58) has been modified, tuned down and now reads:"but the factors that regulate cell migration in each cortical layer are only partially known"

6. Initially (first submission) the authors mention that a small hole in the membrane insert with a pipette tip is necessary to facilitate administration of compounds in the incubation medium. However, this point is removed from the manuscript of the second revision. Could the authors explain why? if this hole is needed it should be mentioned and also shown in the video.

The editors from JoVE asked us previously to move this point to the discussion section. Consequently, I have followed their recommendations. This point has not been removed and you can read it from line 334 to line 336. We do not think that this point is essential for the video.

7. line 132 - Is the N2 supplement diluted from 100x to 1x? please clarify

This point has been clarified and the sentence (line 136) has been modified and reads: …, add N2 supplement (from a X100 stock solution)…

8. line 133 - In what ratio is the DMEM-F12 mixture?
The ratio of the DMEM-F12 mixture is 1:1. The sentence (line 138) has been modified and now reads …(DMEM) nutrient mixture F-12 (1:1).

9. Is there a problem with photo-bleaching of the cell tracker dye? in Fig.3B after 240min it seems that the brightness of the migrating GC is reduced compared to the 0min time point. If the authors observed photo-bleaching they should discuss it.

Cell tracker green is a very stable fluorescent dye. Very rarely, a weak photobleaching can be observed without affecting the tracking of GC. Photobleaching does not only depend on the dye but also on the light intensity. Ten years ago, we used cell tracker green with confocal microscopy and more recently, we switch to confocal macroscopy. In these new conditions, we observed less photobleaching events. As suggested by the referee #2, we underline that confocal macroscopy approach also reduces photobleaching. Line 111, the sentence now reads: …, ensures constant environmental parameters, reduces photobleaching, increases field of view…

10. What are the criteria for the GCs selected for migration analysis? should they be at the border of the EGL/ML? this should be clarify

Depending on the scientific purpose, GC can be observed at different positions in the different cortical layers. For starting experiments, I would suggest to follow GC in the ML where successive control (2 h) and treatment (2 h) periods can be easily performed. In the ML, the

shape of GC with vertically elongated cell body should be considered as a reference to start acquisition.
To satisfy the referee #2 request, we added the following sentences in the discussion section, line 373: "For starting experiments, observation of GC movements in the ML is suggested. Indeed, the shape of GC with vertically elongated cell body should be considered as a reference point to start acquisition with successive control (2 h) and treatment (2 h) periods that can be easily performed in the ML."

Reviewer #3:
Manuscript Summary:
This paper describes the protocol of live imaging using acute cerebellar slices. The protocol allows observation of migrating granule cells for 4-10 hr, which makes it possible to examine the role of molecules that might be involved in neuronal migration. The protocol will of great use for researchers who are interested in the mechanism of neuronal migration in the postnatal brain. The text is well written, and the protocol is written sufficiently in detail and all the steps listed in the procedure are clearly explained. Although the manuscript is acceptable with the current from, I have some suggestions and comments.

Major Concerns:
N/A

Minor Concerns:
1. The authors use macro confocal system. I understand that this is useful to capture many cells at once. However, multipoint scanning confocal microscope is also used for the same purpose. Description of advantage and disadvantage over this method will be useful.

Multipoint scanning confocal microscopes as spinning disk or multi-pinhole confocal system proposed fast acquisitions while reducing the photobleaching and phototoxicity. However, these systems only offer field of view of microscopy set up without allowing easy manipulations and access to the sample as provided by the confocal macroscope approach. In addition, we do not have major problems of photobleaching with the confocal macroscope approach and time resolution (1 acquisition every 30 min) is not a key point. With a motorized stage, mosaic imaging can be considered in multipoint scanning confocal microscopy but time lag and focus drift would be maybe problematic. An interesting solution, that can be suggested to commercial suppliers or laboratories involved in the development of new equipments, is to set up a multipoint scanning confocal macroscope.
To underline the advantage of multipoint scanning confocal microscopes in the context of GC migration, we added line 111: "as proposed by multipoint scanning confocal microscope approach".

2. Readers will be interested to know how the authors judged the health of slices.

As described line 370 to line 372, GC migration is a physiological process and cells have to migrate properly in healthy slices. In addition, shape of cell body and number of labeled cells

are also key parameters to judge the health of slices. In particular, numerous GC with elongated cell body in the molecular layer is one of main health indicator of sagittal cerebellar slices.
To answer to the referee and give additional guidelines to the readers, we added line 372: "In particular, numerous spindle GC in the ML is one of the main health indicator for sagittal cerebellar slices."

Other comments
Line 82: Is "modifify" correctly spelled?
"Modifify" has replaced by "modify"

Line 240: It would be useful to describe the NA of the objective.
The Numerical Aperture of the objective was indicated line 349. As proposed by the referee, NA= 0.234 was also added line 245.

Line 279: Insert a space after ML.
A space after ML was added line 284.

Line 349: "compare"
should read as "compared.
Compare as been correct to compared line 360

As a result of the review process, we would like to thank the reviewers for their recommendations and we feel that our manuscript has been improved substantially.

Thank you for your time in consideration of this revised manuscript. With best regards.

Ludovic Galas

TITLE:
Ex vivo imaging of postnatal cerebellar granule cell migration using confocal macroscopy

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KEYWORDS:
Neuroscience, developmental biology, cerebellum, interneuron migration, cerebellar granule cell, neuropeptide, PACAP, serine protease, tPA

SHORT ABSTRACT:
During postnatal cerebellum development, immature granule cells originating from the

germinal zone exhibit distinct modalities of migration to reach their final destination and to establish neuronal networks. This protocol describes the preparation of cerebellar slices and the confocal macroscopic approach used to investigate the factors that regulate neuronal migration.

LONG ABSTRACT:

During postnatal development, immature granule cells (excitatory interneurons) exhibit tangential migration in the external granular layer, and then radial migration in the molecular layer and the Purkinje cell layer to reach the internal granular layer of the cerebellar cortex. Default in migratory processes induces either cell death or misplacement of the neurons, leading to deficits in diverse cerebellar functions. Centripetal granule cell migration involves several mechanisms, such as chemotaxis and extracellular matrix degradation, to guide the cells towards their final position, but the factors that regulate cell migration in each cortical layer are only partially known. In our method, acute cerebellar slices are prepared from P10 rats, granule cells are labeled with a fluorescent cytoplasmic marker and tissues are cultured on membrane inserts from 4 to 10 h before starting real-time monitoring of cell migration by confocal macroscopy at 37 °C in the presence of CO_2. During their migration in the different cortical layers of the cerebellum, granule cells can be exposed to neuropeptide agonists or antagonists, protease inhibitors, blockers of intracellular effectors or even toxic substances such as alcohol or methylmercury to investigate their possible role in the regulation of neuronal migration.

INTRODUCTION:

In the developing cerebellum, eight different types of neurons are produced sequentially between the second embryonic week and the second postnatal week in rodents[1]. Originating initially from, a primary germinal zone, immature granule cells (GC) are the last neurons to be produced from the external granular layer (EGL), a secondary germinal zone[2]. During the first three postnatal weeks, the cerebellar cortex is a foliated structure organized in four layers including the EGL, the molecular layer (ML), the Purkinje cell layer (PCL) and the internal granular layer (IGL) (Figure 1). Through centripetal migration, immature GCs, glutamatergic interneurons, reach the IGL within approximately 2 days. By the third postnatal week, the EGL disappears and the IGL constitutes what is called the granular layer (GL) in the adult cerebellum. In the GL, GCs receive excitatory synaptic inputs from mossy fibers and unipolar brush cells, and inhibitory synaptic inputs from Golgi cell axons. In the ML, GC axons make excitatory synapses with GABAergic neurons including Purkinje cells, basket cells, stellate cells, and Golgi cells[2].

Real-time observation of cell movement in acute cerebellar slices obtained from early postnatal rodents demonstrates that GCs modify their shape concomitantly with changes in the modality and speed of migration during their route in the cerebellar cortex[3]. During first two postnatal weeks, GC precursors actively proliferate at the top of the EGL. In the middle part of the EGL, postmitotic GCs migrate tangentially in the direction of their larger process. At the EGL-ML border, GCs slow their movement, the cells begin to enter a short vertical descending process into the ML. In the ML, GCs have a vertically elongated cell body, a thin trailing process and a more voluminous leading process, and migrate radially along the Bergmann glial fibers. In the

PCL, GCs stop their movement but after a prolonged stationary phase (~2 h), they cross the PCL-IGL border. In the IGL, GCs migrate towards the bottom of the layer in the absence of glial fiber support. Once the tips of the leading process approach the IGL-white matter (WM) border, GCs slow and stop their movement. Transverse sections of the cerebellum are preferred for tangential migration studies in the EGL while sagittal slices are dedicated to radial migration in the ML, PCL and IGL. Some regulatory factors of GC movements including neuropeptides (e.g., somatostatin, PACAP) have been identified so far but the complete mechanisms involved in the spatio-temporal control of GC migration in each cortical layer are still largely unknown[1,4,5,6].

GC migration has been studied during the last 20 years through video- and confocal microscopy using either transmitted light illumination for isolated cultured cells or fluorescence detection for acute cerebellar slices. Initially lipophilic DiI, and more recently "Cell Tracker" dyes and cell-expressed fluorescent proteins were used for confocal or two-photon microscopy[7,8]. Successful experiments depend on a number of specific procedures that make the protocol simple but not easy. In particular, acute slices have to be stabilized during observations generally with a home-made nylon mesh network[9]. The intensity of light illumination has to be as low as possible to avoid phototoxicity and photobleaching as proposed by multipoint scanning confocal microscope approach. In addition, temperature and CO_2 are key environmental parameters since instability may affect neuronal migration. To facilitate and refine the experimental procedures, we have developed a confocal macroscopy protocol that limits slice movements, ensures constant environmental parameters, reduces photobleaching, increases field of view (on the order of mms) and consequently the number of cells (dozens) that can be tracked through image analysis. Thus, 180 µm thick slices are cultured on membrane inserts, and 6-well plates are directly transferred under a 2x motorized objective of a commercial confocal macroscope equipped with a large incubation chamber, temperature and CO_2 controllers and a vibration control system. Time-lapses and z-stacks are then performed over several hours and pharmacological tools or bioactive molecules can be added or delivered in the incubation medium. This method could also be adapted to study the migration of the other types of neurons in the cerebellum or cerebrum at different developmental stages.

PROTOCOL:

Animals (male or female Wistar rats) were born and bred in an accredited animal facility (approval B.76-451-04), according to the French guide for the care and use of laboratory animals. Experiments were conducted under the supervision of authorized investigators (M.B, D.V. and L.G.) in accordance with the European Community Council Directive (2010/63/UE of September 22, 2010) and the French Ministry of Agriculture.

1. Preparation of media and tools

1.1) In a biological safety cabinet, prepare 1X Hank's BSS (HBSS) in sterile water from 10X stock solution containing $CaCl_2$ (1.85 g/L) /$MgSO_4$ (0.9767 g/L) without $MgCl_2$. Add $NaHCO_3$ (350 µg/mL) to 1X solution of HBSS.

1.2) In a biological safety cabinet, add N2 supplement (from a 100X stock solution) and penicillin (100 units/mL) -streptomycin (0.1 mg/mL) solution to Dulbecco's modified eagle medium (DMEM) nutrient mixture F-12 (1:1).

1.3) In sterile conditions, prepare an aliquot (25 µL, 2 mM) of the cytoplasmic fluorescent dye called Cell Tracker Green in DMSO (1.075 mL for 1 mg). Dilute one aliquot in 5 ml of DMEM in a 15 mL conical tube.

1.4) Prepare filled ice-bucket to keep media at 4 °C.

1.5) Decontaminate laboratory bench tops and tools with 70% ethanol.

2. Dissection of cerebellum from P10 rat

2.1) Rapidly decapitate rat pups (P10) with curved operating scissors behind ears in order to get the beginning of the spinal cord.

2.2) At the back-side of the decapitated head, make midline incision of the skin from the neck to the nose with fine iris scissors and separate the skin from the skull with fine iris scissors and Dumont #3 forceps.

2.3) Use fine iris scissors to delicately make two lateral incisions from the base to the rostral region of the skull. Remove the dissected skull with two #3 forceps. Detach the brain from any adherence with the skull using the same forceps.

2.4) Transfer the brain with the spoon end of a spatula to Petri dish (Ø 35 mm) containing 2 mL of ice-cold HBSS medium.

2.5) Put the Petri dish containing the brain in a larger Petri dish (Ø 100 mm) full of ice and transfer to the stage of the stereomicroscope.

2.6) Under a stereomicroscope, isolate the cerebellum from the brain by dilaceration using two #3 forceps. Similarly, remove the residual spinal cord and the pial-membrane.

2.7) Transfer the cerebellum in a new HBSS-filled Petri dish (Ø 35 mm, 2 mL) with the spoon end of a spatula and keep on ice.

3. Preparation of acute cerebellar slices

3.1) Under the stereomicroscope, cut the cerebellum between the vermis and the right hemisphere as indicated by the two heads arrow in Figure 2A with a standard scalpel handle #3 solid and a #15 surgical blade.

3.2) Put one drop of cyanoacrylate glue on the vibratome specimen disc and wait 15-25 s to

eliminate toxic solvent vapors.

3.3) Collect the cut cerebellum with the spoon end of a spatula and remove excess HBSS with clean paper towels.

3.4) Bring the cerebellum close to the specimen disc. Fix the cut edge to the specimen disc and wait 10 s.

3.5) Insert the specimen disc into the buffer tray with the manipulator, and rotate it so the transverse axis of the cerebellum is perpendicular to the knife holder. Fix the specimen disc with an Allen key and fill gently the buffer tray with HBSS medium until the cerebellum is covered.

3.6) Load crushed ice into the cooling bath.

3.7) Clean the blade three times with 70% ethanol to eliminate any oil.

3.8) Insert the blade into the knife holder and secure with clamping screw.

3.9) Place the blade edge right behind the rear edge (from user's view) of the specimen and define it as a starting point. Use forward command to define the ending point after the front edge of the specimen.

3.10) Select sectioning speed at 2.5 and sectioning frequency at 8. Select trimming thickness at 180 µm. Start tissue sectioning.

3.11) Pick up each section using a wide-bore glass truncated Pasteur pipette and transfer to HBSS-containing Petri dish (Ø 35 mm) kept on ice.

3.12) Using two #5 forceps, remove the meninges carefully from the cerebellum when interfering with the blade. Collect a maximum of 5 slices per cerebellum (Figure 2B, C).

3.13) Remove the meninges from the cerebellar slices carefully with two #5 forceps under the stereomicroscope and separate the lobules gently for better probe loading.

4. Fluorescent staining of living interneurons

4.1) Transfer cerebellar slices with a wide-bore glass truncated Pasteur pipette to a 6-well plate (max 3 slices/per well). Aspirate the HBSS medium.

4.2) Incubate slices (3 max) in 5 mL of loading solution of the fluorescent dye (10 µM).

4.3) To protect from light, cover the microplate with aluminum foil. Put it on a gyro-moving table at 35 rpm for 10 min at room temperature to facilitate cell labeling.

4.4) Transfer slices on the membrane of a Transwell insert (3.0 µm pore size; Figure 2D) with a wide-bore glass truncated Pasteur pipette. Aspirate the loading medium with pipette.

4.5) Remove the insert and fill the well with 1.9 mL of DMEM. Replace the insert and add 100 µL of DMEM on top of the slice to cover the tissue.

4.6) Place the plate containing the culture inserts in the incubator chamber (37 °C, 5% CO_2) for 2 h which is sufficient to observe GCs in the ML. Lie tissues flat to allow attachment on the insert membrane (Figure 2E). Ensure that slices are not drying.

5. *Ex vivo* imaging through confocal macroscopy

5.1) Transfer the plate without the plastic lid into an incubator attached to the stand of a confocal macroscope. Place a glass cover on the plate insert of the macroscope. Keep the temperature of the chamber at 37.0 °C ± 0.5 °C, and supply the slices with constant gas flow (95% O_2, 5% CO_2) through the plate insert to maintain the pH constant. Wait for 2 additional hours before time-lapse experiment.

5.2) To visualize GC migration in the tissue slices, illuminate the preparation with a 488-nm wavelength light by means of a laser diode through a confocal laser scanning macroscope equipped with a X2 dry objective (working distance: 39 mm, diameter: 58 mm, NA=0.234), and detect fluorescence emission from 500 to 530 nm.

5.2.1) To finely resolve the movement of GCs, acquire images with an additional optical zoom factor of 1.5 to 2.0. Collect images of GNs in a single focal plane or up to 10 different focal planes along the z-axis every 30 min for up to 12 hours.

5.3) When necessary, remove the glass cover and add small volumes (1-10 µL) of biological activators or inhibitors in DMEM with a 10 µL pipette to study their effect on GC migration.

6. Cell tracking

6.1) For each time of the movie, perform z-stack projection through the ecart-type mode in ImageJ. Modulate the contrast and the brightness levels of the successive images to facilitate the identification and the tracking of labeled GCs. Map manually each position on the reference snapshot (at t=0).

6.1.1) Use the “Manual tracking” plugin in the Analyse Particle Menu and determine by clicking the gravity point of each cell body during time-lapse. Export the raw tracking data in a spreadsheet.

6.2) Reorganize the exported raw tracking data from ImageJ with a smart home-made program (http://primacen.fr, written in PHP code) that identify each cell and associated

positions. Using the program, calculate the total traveled distance and the average speed of migration for each cell. Classify and compare characteristics of cell migration in control and treatment conditions under appropriate filters using the same program.

REPRESENTATIVE RESULTS:

In the early postnatal cerebellum, GCs exhibit significant changes in their mode and speed of migration as they cross different cortical layers[1] (Figure 1). This section illustrates examples of results that can be obtained by studying GC migration in their natural cellular milieu. P10 rat cerebellar tissue slices labeled with a green fluorescent dye are examined under a confocal macroscope (Figure 3A) and we show that GCs migrate radially in the ML with an average speed of 18 µm/h (Figure 3B, C). To date, the role of interactions/communications between neuronal and glial cells including the regulatory factors and molecular mechanisms involved in the control of cell migration in each cortical layer are largely unknown. Consequently, the main issue is to identify neuropeptides, neurotransmitters, neurotrophins and extracellular matrix components that could play a role in these cortical layer-specific changes of the speed during their migration process. Pituitary adenylate cyclase-activating polypeptide (PACAP) is detected mainly in the PCL, but also in the ML and the IGL during the first two postnatal weeks in rodents[7,10,11]. Application of PACAP38 (10^{-6} M) to the culture medium resulted in a 79% speed decrease of the GC in the ML. For example, the migration velocity of GCs in the ML dropped from 11.9 µm/h in control conditions to 2.5 µm/h after administration of PACAP38 (Figure 4A). Tissue-type plasminogen activator (tPA) is a member of the proteolytic cascade that leads to the degradation of the extracellular matrix (EM) components such as cell adhesion molecules or laminin[12,13]. tPA and plasminogen, a substrate of tPA, are detected in cortical layers during development of the postnatal cerebellum[14,15,16]. Administration of PAI-1 (10^{-7} M), an inhibitor of endogenous tPA, reduced by 78% the GC migration in the ML. For example, GCs reduced migration speed in the ML from 19.2 µm/h in control conditions to 4.2 µm/h after addition of PAI-1 (Figure 4B). These results indicate that PACAP exerts a direct inhibitory effect on GC movements and that the serine protease tPA facilitates the migration of GCs in the ML of the developing rat cerebellum.

Figure 1. 3D representation of GC migration in the postnatal cerebellar cortex. 1-4, Extension of GC processes and tangential migration in the EGL. **5**, Radial migration in the ML along Bergmann glial fibers. **6**, Transient stationary phase in the PCL. **7**, Glial-independent radial migration in the IGL. **8**, Completion of GC migration in the IGL. **GC**, granule cell, in red; **EGL**, external granular layer; **B**, Bergmann glia, in dark purple; **G**, Golgi cell, in yellow; **cf**, climbing fibers, in blue; **g**, postmigratory granule cells, in light green; **IGL**, internal granular layer; **mft**, mossy fiber terminal, in dark green; **ML**, molecular layer; **P**, Purkinje cell, in light purple; **PCL**, Purkinje cell layer. This figure has been modified [5].

Figure 2. *Ex vivo* culture of P10 cerebellar slices. (A) Dissected cerebellum from P10 rat. Scale bar = 6 mm. **(B)** Micrograph of living 180 µm-thick cerebellar slice through stereomicroscopy. Scale bar = 3 mm. **(C)** At a higher magnification, the four cortical layers (**EGL**, **ML**, **PCL**, **IGL**) of the cerebellum are already distinguishable. Scale bar = 1 mm. **(D)** After fluorescent labeling, tissue slices are placed on culture inserts (24 mm diameter) into a 6-well plate. **(E)** Schematic

representation of a culture insert with tissue culture treated polyester membrane.

Figure 3. Dynamic migration of GCs in the cortical layers of rat P10 cerebellum. (A) Macroconfocal view (xyz, 2D projection) of a P10 rat cerebellar slice in which GCs are labeled with a green cytoplasmic fluorescent dye. Scale bar = 75 µm. **(B)** Time-lapse imaging showing GC movements in the ML by confocal macroscopy for 4 h in control conditions. Asterisk (*) symbol marks the GC soma. Elapsed time (in min) is indicated on the bottom of each photomicrograph. Scale bar = 10 µm. **(C)** Sequential changes in the distance traveled by GC soma.

Figure 4. Effect of neuropeptide and protease inhibitor of GC migration. (A) GC was tracked in the ML by confocal macroscopy for 2 h in control conditions and then for 2 h in the presence of Pituitary adenylate cyclase-activating polypeptide (PACAP). **(B)** GC was tracked in the ML by confocal macroscopy for 2 h in control conditions and then for 2 h in the presence of plasminogen activator inhibitor-1 (PAI-1).

DISCUSSION:

This protocol describes the culture of acute P10 rat cerebellar slices in the Transwell system and the fluorescent labeling of GC with a green fluorescent dye to study cell migration during postnatal development through confocal macroscopy. This protocol allows observations of cell migration for a period up to 12 h and testing of the possible roles of regulating factors in migration including agonists or antagonists of neuropeptides, enzyme inhibitors, cell signaling modulators or toxic substances during the experiment. A small hole in the membrane insert with a pipette tip is necessary to facilitate administration of compounds in the incubation medium. A home-made curved tip pipette can be used to facilitate the delivery of solution.

One issue of cell migration studies on living tissue slices is that the movements of the tissue itself can make cell tracking difficult. Whereas previous approaches have proposed to gently stabilize slices with a nylon mesh or a thin layer of rat tail collagen[7,17], a major advantage of this technology is the simple and direct transfer of a 6-well culture plate containing cerebellar slices on membrane inserts from the CO_2 incubator under the objective of a confocal macroscope. Integrated temperature and CO_2 controllers also provide appropriate and constant environmental parameters essential for cell migration[9]. Therefore, culture conditions are kept during observations and tissue movements are minimized since slices are well attached to the membrane insert. Stabilization of the tissue is verified by following the position of slice edges or Purkinje cells that should be fixed references during acquisition. In addition, cerebellar slices (between 12 and 18) distributed in the 6 wells of the plate can be quickly observed in detail with a motorized stage and an optical zoom. Due to the large working distance (X2, 39 mm) of the dry objective, epi-observation is free of immersion and administration of compounds in the culture medium is much easier. Therefore, environmental parameters and culture support similarities in CO_2 incubator and confocal macroscopy lead to maximum conservation of the biological sample.

Another advantage of the protocol is the large field of view and consequently the large number

of cells that can be observed simultaneously. For example, we have previously determined that the density of fluorescent GCs with radial migration in the ML was (1124 ± 138) cell/mm^2 [18]. Confocal macroscopy (X2, NA=0.234) has a lower lateral resolution compared to confocal microscopy (40X, NA=1.25) but cell bodies of GCs can be easily tracked and the average speed of migration is comparable between the two technological approaches[7,18].

Besides technical improvement for image acquisitions, the quality of tissue slices and the quality of labeling are key points for successful experiments. Always keep media and tissues on ice during dissection processes, eliminate oil on vibratome blades and do not utilize slices of tissue in contact with glue. Sagittal and transverse sections are adapted to radial and tangential migration respectively. Use different lengths of incubation for appropriate detection in the different cortical layers of the cerebellum. Long incubation times (up to 8 h) are necessary to detect the migration of numerous GCs in the PCL and the IGL. Since GC migration is a physiological process during specific spatio-temporal windows, the positive control is that the cells have to migrate properly. In particular, numerous spindle GC in the ML is one of the main health indicator for sagittal cerebellar slices. For starting experiments, observation of GC movements in the ML is suggested. Indeed, the shape of GC with vertically elongated cell body should be considered as a reference point to start acquisition with successive control (2 h) and treatment (2 h) periods that can be easily performed in the ML.

Fluorescent dyes like the Cell Tracker family or fluorescent proteins expressed through genetic constructs can be used as tracers for cell migration studies. Due to the slow kinetics of GC migration (1 stack every 30 min), multicolor experiments can also be performed in sequential mode since 4 lasers beams (405, 488, 532 and 633 nm) are available on the system. Considering centripetal and centrifugal radial migration, tracking of other interneurons can also be realized[18]. In particular, less numerous cell types can be more easily localized with a large field of view. Finally, this protocol can be used to study cell migration at other stages of cerebellar development but also other brain areas.

DISCLOSURES:
The authors have nothing to disclose.

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REFERENCES:
1. Komuro, Y., Kumada, T., Ohno, N., Foote, K.D. & Komuro, H. Migration in the Cerebellum. *Cellular Migration and Formation of Neuronal Connections: Comprehensive Developmental Neuroscience*. Volume **2**, 281–297, doi: 10.1016/B978-0-12-397266-8.00030-2 (2013).
2. Altman, J. & Bayer, S. A. Development of the cerebellar system in relation to its

evolution, structure, and functions. CRC Press, Boca Raton FL (1997).
3. Komuro, H. & Rakic, P. Distinct modes of neuronal migration in different domains of developing cerebellar cortex. *Journal of Neuroscience.* **18** (4), 1478–1490 (1998).
4. Komuro, H. & Yacubova, E. Recent advances in cerebellar granule cell migration. *Cellular and Molecular Life Sciences.* **60** (6), 1084–1098 (2003).
5. Fahrion, J.K. *et al.* Rescue of neuronal migration deficits in a mouse model of fetal Minamata disease by increasing neuronal Ca^{2+} spike frequency. Proceedings of National Academy of Sciences USA. **109** (13), 5057-5062, doi: 10.1073/pnas.1120747109 (2012).
6. Raoult, E., *et al*. Pituitary adenylate cyclase-activating polypeptide (PACAP) stimulates the expression and the release of tissue plasminogen activator (tPA) in neuronal cells: involvement of tPA in the neuroprotective effect of PACAP. *Journal of Neurochemistry.* **119** (5), 920–931, doi: 10.1111/j.1471-4159.2011.07486.x (2011).
7. Cameron, D. B. *et al.* Cerebellar cortical-layer-specific control of neuronal migration by pituitary adenylate cyclase-activating polypeptide. *Neuroscience* **146** (2), 697–712 (2007).
8. Renaud, J., *et al*. Plexin-A2 and its ligand, Sema6A, control nucleus-centrosome coupling in migrating granule cells. *Nature Neuroscience*. **4**, 440-449, doi: 10.1038/nn2064 (2008).
9. Komuro, H. & Rakic, P. Dynamics of granule cell migration: a confocal microscopic study in acute cerebellar slice preparations. *Journal of Neuroscience.* **15** (2), 1110–1120 (1995).
10. Nielsen, H. S., Hannibal, J. & Fahrenkrug, J. Expression of pituitary adenylate cyclase activating polypeptide (PACAP) in the postnatal and adult rat cerebellar cortex. *Neuroreport* **9** (11), 2639–2642 (1998).
11. Hannibal, J. Pituitary adenylate cyclase-activating peptide in the rat central nervous system: an immunohistochemical and in situ hybridization study. *Journal of Comparative Neurology.* **453** (4), 389–417 (2002).
12. Garcia-Rocha, M., Avila, J. & Armas-Portela, R. Tissue-type plasminogen activator (tPA) is the main plasminogen activator associated with isolated rat nerve growth cones. *Neuroscience Letters.* **180** (2), 123–126 (1994).
13. Ware, J. H., DiBenedetto, A. J. & Pittman R. N. Localization of tissue plasminogen activator mRNA in the developing rat cerebellum and effects of inhibiting tissue plasminogen activator on granule cell migration. *Journal of Neurobiology.* **28** (1), 9–22 (1995).
14. Friedman, G. C. & Seeds, N.W. Tissue plasminogen activator mRNA expression in granule neurons coincides with their migration in the developing cerebellum. *Journal of Comparative Neurology.* **360** (4), 658–670 (1995).
15. Seeds, N. W., Siconolfi, L. B. & Haffke, S. P. Neuronal extracellular proteases facilitate cell migration, axonal growth, and pathfinding. *Cell and Tissue Research.* **290** (2), 367–370 (1997).
16. Basham, M. E. & Seeds, N. W. Plasminogen expression in the neonatal and adult mouse brain. *Journal of Neurochemistry.* **77** (1), 318–325 (2001).
17. Saha, B., Ypsilanti, A.R., Boutin, C., Cremer, H. & Chédotal, A. Plexin-B2 regulates the proliferation and migration of neuroblasts in the postnatal and adult subventricular zone. *Journal of Neuroscience.* **32** (47), 16892-16905, doi: 10.1523/JNEUROSCI.0344-12 (2012).
18. Raoult, E. *et al*. Cortical-layer-specific effects of PACAP and tPA on interneuron migration during post-natal development of the cerebellum. *Journal of Neurochemistry*. **130** (2),

241–254, doi: 10.1111/jnc.12714 (2014).
19. Bourgault S. *et al*. Molecular and conformational determinants of pituitary adenylate cyclase-activating polypeptide (PACAP) for activation of the PAC1 receptor. *Journal of Medical Chemistry.* **52** (10), 3308–3316, doi: 10.1021/jm900291j (2009).

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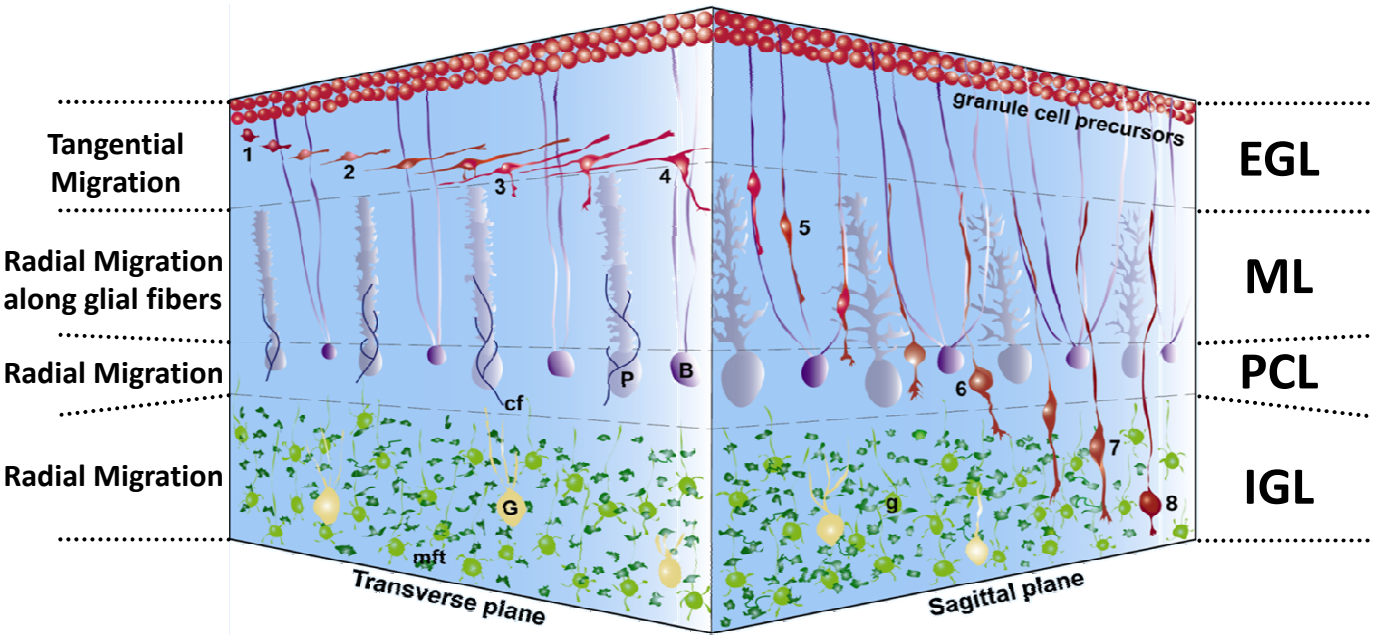

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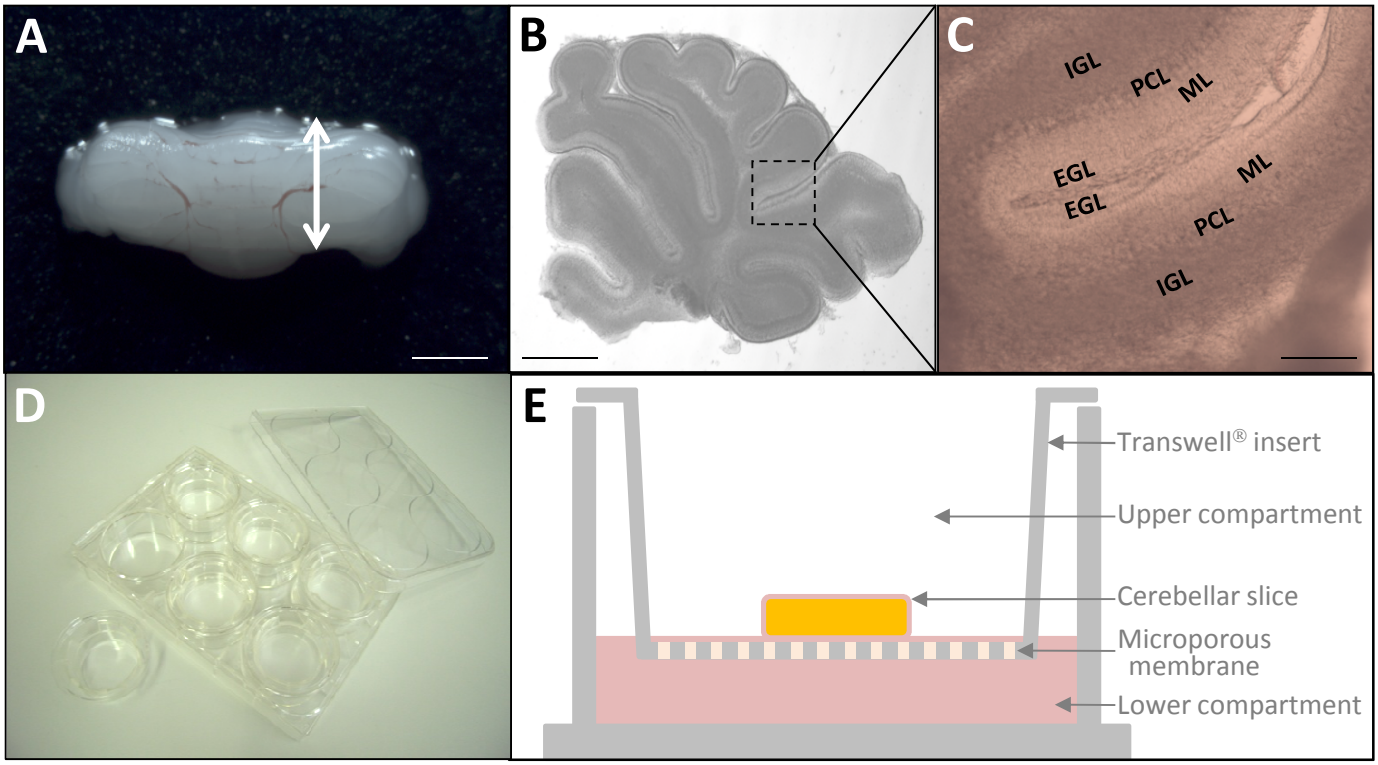

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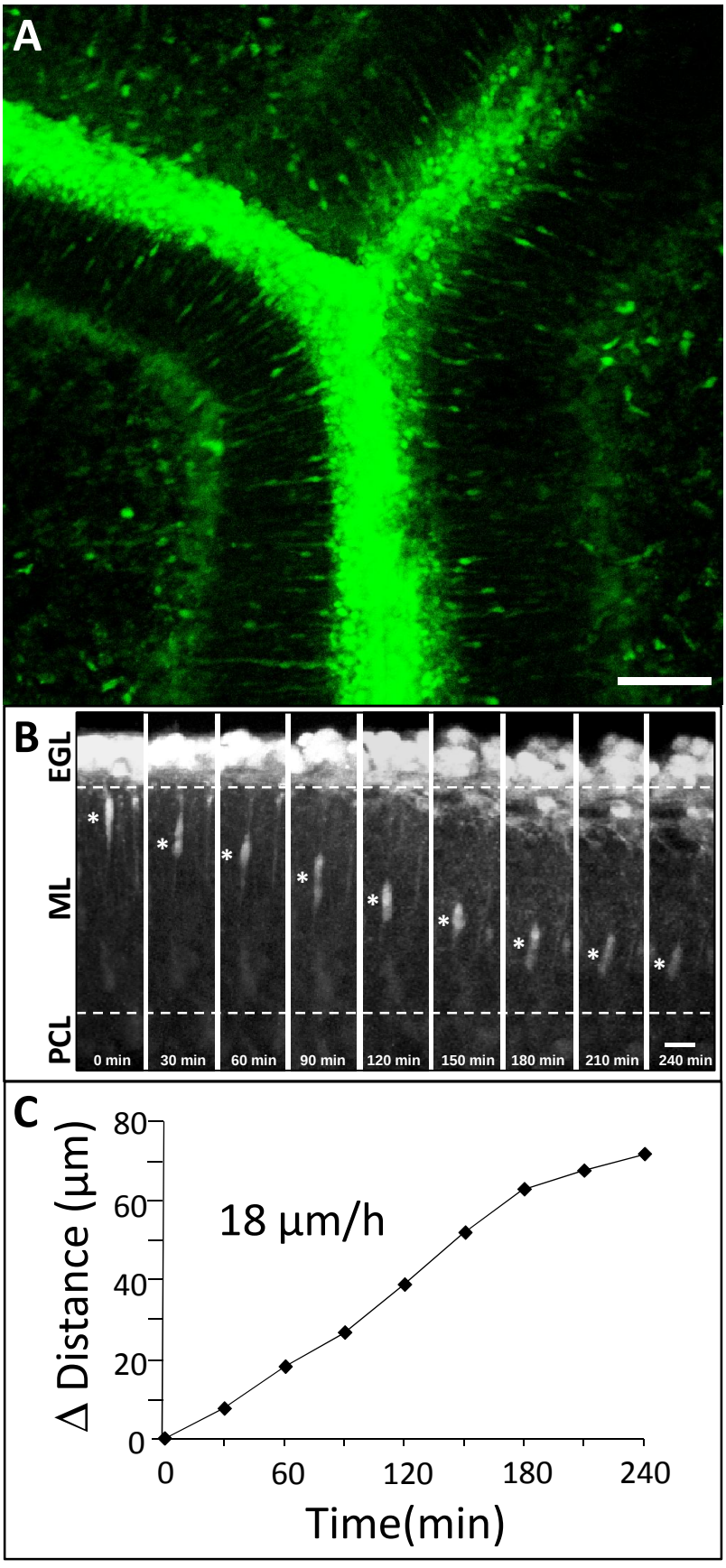

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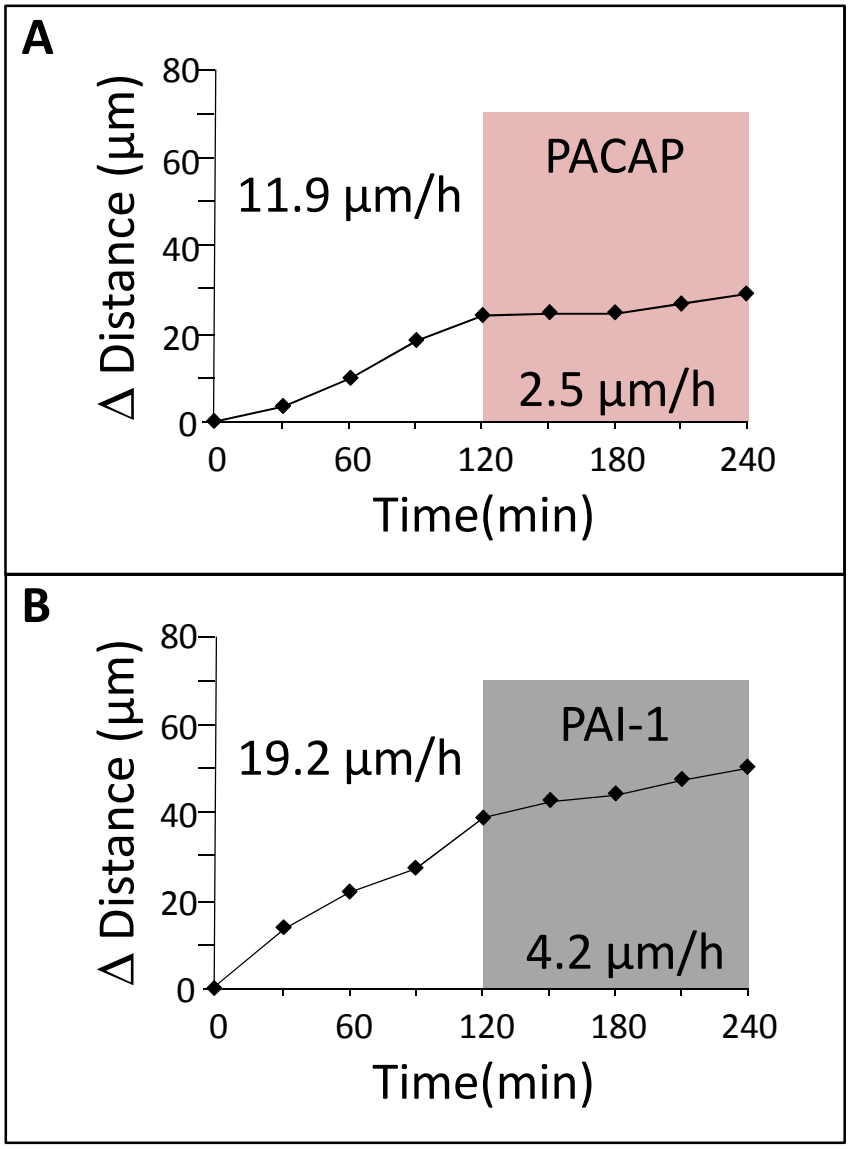

Figure 4

Excel Spreadsheet- Table of Materials/Equipment

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Name of Product/Material/Equipment	Company	Catalog Number
Dulbecco's modified eagle medium (DMEM) nutrient mixture F-12	Sigma-Aldrich	D8437
Hank's balanced salt solution 10X	Sigma-Aldrich	H1641
PACAP38	INRS, Canada	
PAI-1	Calbiochem	528208
N-2 supplement	ıer Scientific / Gibco/ invitro	O973
cyanoacrylate glue	Loctite	
Cell Tracker Green CMFDA	Invitrogen	C2925
Polyester Transwell-Clear inserts	Corning	3452
Penicillin-Streptomycin	Sigma-Aldrich	P0781
6-well cell culture cluster	Corning	3516
DMSO	Fisher Scientific	BP231-100
Tissue culture dish 35 mm diameter	BD Falcon	353004
Tissue culture dish 100 mm diameter	Thermo SCIENTIFIC	130182
Polypropylen tube (15 ml)	BD Falcon	352096
Ethanol 70%	Fisher Chemical	E/0800DF/21
biological safety cabinet fume hood	Thermo Scientific	MSC9 Class II A2
adjustable-volume pipette (0,5-10 µL)	Eppendorf	4910 000.018
gyro-rocker, SSL3	Stuart	
CO_2 incubator, Hera Cell 150	Thermo Scientific	
vibrating blade microtome, VT1000S	Leica Microsystems	
confocal macroscope, TCS LSI	Leica Microsystems	
temperature controller	PeCon	
CO_2-controller	PeCon	
Stereomicroscope, M205 C	Leica Microsystems	
Operating scissors, curved, blunt/blunt	Medicon	03.03.17
Hardened fine iris scissors, straight, sharp/sharp	FST	149090-11
Dumont #3 and #5 forceps	FST	11293-00 and 11252-20

Vibratome injector blades/single edge	Leica Microsystems	39053250
standard scalpel handle #3 solid	FST	10003-12
surgical blade #15	Swann-Morton	205

	Comments/Description
Bourgault et al., 2009[19]	

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