INTRODUCTION

In the developing nervous system, the selectivity of the neurotrophins for the survival of particular populations of neurons during development depends not only on the specific spatiotemporal pattern of expression of the ligand in the target tissue but also of its corresponding Trk receptors in the neurons. However, while Trk receptors are structurally similar, their specific binding can lead to the activation of different signaling pathways with distinct impacts on target innervation and neuronal survival in vivo (Postigl et al., 2002; Nikolopoulos et al., 2010). Although significant progress has been made in our understanding of the specificity of the downstream effectors different Trk receptors are using, the molecular basis supporting these differences remains not well understood.

Spiral ganglion neurons (SGNs) constitute the first relay of the acoustic neuroanatomical ascending pathway between the sensory receptors – the inner and outer hair cells (IHCs/OHCs) of the organ of Corti – of the mammalian cochlea and the central nervous system (Navagam et al., 2011). SGNs are divided in two types: type I neurons innervating specifically the IHCs, representing about 90% of the neuronal population while the type II neurons, representing the remaining 10%, contact the OHCs and are thought to provide an integrated afferent feedback loop and to amplify both cochlear sensitivity and frequency discrimination (Geleoc and Holt, 2003). The development of SGNs starts at mid gestation and is completed during the two first postnatal weeks in rodents. Among neurotrophins, brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT3) through their respective receptors, TrkB and TrkC, have been shown to play a central role in regulating the development of SGNs (Fritsch et al., 2005; Defourny et al., 2011). Hence, they are expressed and secreted by the developing otic sensory epithelium and act on neuronal TrkB and TrkC to control their survival and the growth of their projections towards the hair cells (Farinas et al., 2001; Defourny et al., 2011). As such, mice with knockouts of the NT3 or the BDNF genes (or of their specific receptors) have shown a marked reduction, of about 80–90% (NT3-null) and 10% (BDNF-null), in SGN number (Farinas et al., 1994; Ernfors et al., 1995; Tessler et al., 1997; Wiesch et al., 1999). This differential sensitivity suggested a specific survival function of NT3 and BDNF for type I and II SGNs, respectively. Although subsequent studies have shown that the specificity of neurotrophin-dependent neurotrophins are key players of neural development by controlling cell death programs. However, the signaling pathways that mediate their selective responses in different populations of neurons remain unclear. In the mammalian cochlea, sensory neurons differentiate perinatally into type I and II populations both expressing TrkB and TrkC, which bind respectively brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT3). How these two neuronal populations respond differentially to these two neurotrophins remains unknown. Here, we report on rat the segregation of the nuclear factor-κB (NF-κB) subunit p65 specifically within the type II population postnatally. Using dissociated cultures of embryonic and postnatal spiral ganglion neurons, we observed a specific requirement of NFkB for BDNF- but not NT3-dependent neuronal survival during a particular postnatal time window that corresponds to a period of neuronal cell death and hair cell innervation refinement in the developing cochlea. Consistently, postnatal p65 knockout mice showed a specific decreased number in type II spiral ganglion neurons. Taken together, these results identify NFkB as a type II neuron-specific factor that participates in the selective survival effects of BDNF and NT3 signaling on developing spiral ganglion neurons.

Keywords: spiral ganglion neurons, neurotrophins, neuronal survival, development, NF-κB, cochlea
survival for the two types of SGNs may also depend on the baso-apical location within the cochlea (Frittsch et al., 1997; Farinas et al., 2001), the analysis of a specific TrkB mutant, which lacks part of the TrkB intracellular signaling pathway and survives until adulthood, confirmed this essential role of the BDNF-TrkB signaling in controlling the survival of type II SGNs (Schimmang et al., 2003). This targeted survival promoting action of BDNF was also observed in vitro where treatment of postnatal SG explants with BDNF rescued essentially the type II population in the absence of organ of Corti-derived endogenous survival-promoting factors (Barclay et al., 2011). However, TrkB and TrkC are expressed in all SGNs, regardless of their type, and both specific ligands are located within the hair cells during development (Furinas et al., 2001), which is not consistent with a differential localization of neurotrophin support but rather suggests that the two types of SGNs are endowed with distinct molecular signaling machineries that are responsible for the preferential survival promoting action of NT3 and BDNF on the type I and II SGNs.

Nuclear factor-κB (NFκB) is an inducible transcription factor that acts as a dimer whose prototype is p50/p65, with p65 containing the transactivation domain. p50/p65 is abundant in a wide range of functions including differentiation and survival (Meffert and Baltimore, 2005). In its silent state, p50/p65 acts as a dimer whose prototype is p50/p65, with p65 containing the transactivation domain. p50/p65 is abundant in all SGNs, regardless of their type, and both specific ligands are located within the hair cells during development (Furinas et al., 2001), which is not consistent with a differential localization of neurotrophin support but rather suggests that the two types of SGNs are endowed with distinct molecular signaling machineries that are responsible for the preferential survival promoting action of NT3 and BDNF on the type I and II SGNs.

To examine the role of p65 in SGNs, viable Mouse lines were group-housed in the animal facility of the University of Liege (Meffert and Baltimore, 2005). In its silent state, p50/p65 containing the transactivation domain. p50/p65 is abundant in all SGNs, regardless of their type, and both specific ligands are located within the hair cells during development (Furinas et al., 2001), which is not consistent with a differential localization of neurotrophin support but rather suggests that the two types of SGNs are endowed with distinct molecular signaling machineries that are responsible for the preferential survival promoting action of NT3 and BDNF on the type I and II SGNs.

**Primary Cultures of SGNs**

Experiments were performed on Wistar rat SGNs from embryonic day (E) 18 to postnatal day (P) 8 rats. Rat species was chosen here as their cochlea is not or incompletely ossified at the ages analyzed, and SG from rat cochlea can be easily isolated. Rats were euthanized and decapitated. Mandibles were removed, bullect exposed and temporal bones excised and transferred into Petri dishes containing phosphate buffered saline (PBS) supplemented with glucose (6 g/l). With the aid of a dissecting microscope and watchmaker’s forceps, the cochlea was isolated and its SG excised. SG were incubated for 40 min at 37°C in a papanicolaou solution (1.5 ml of papain at 1 mg/ml, 0.5 ml of DNAse at 0.1% 20 SG/2 ml solution). Enzymatic activity was terminated by adding ovobumin (0.5 ml of a 10 mg/ml solution). The explants were then washed with DMEM supplemented with N1 additives (Bottenstein and Sat0, 1979) and glucose (6 g/l, final concentration). Mechanical dissociation of the SG tissues was achieved by triturating with a siliconised Pasteur pipette. For cell survival experiments, the resulting ganglion cell suspension was seeded in 96-well plates (Nunc, Roskilde, Denmark) previously sequentially coated with poly-L-ornithine (0.1 mg/ml in 15 mM borate buffer) for 1 h and laminin (10 µg/ml in PBS) for 2 h at 37°C. Cells were maintained at 37°C in a humidified atmosphere of 5% CO2/95% air. Neurotrophins were purchased from Peprotech (Rocky Hill, NJ, USA), sulfasalazine, from Sigma (St Louis, MO, USA) and SN-50, from Calbiochem (San Diego, CA, USA).

**Immunostainings**

For immunohistochemistry, rat cochleae were fixed in 4% paraformaldehyde at 4°C overnight, decalcified (from P5 onward) by trituration with a siliconised Pasteur pipette. For cell survival experiments, the resulting ganglion cell suspension was seeded in 96-well plates (Nunc, Roskilde, Denmark) previously sequentially coated with poly-L-ornithine (0.1 mg/ml in 15 mM borate buffer) for 1 h and laminin (10 µg/ml in PBS) for 2 h at 37°C. Cells were maintained at 37°C in a humidified atmosphere of 5% CO2/95% air. Neurotrophins were purchased from Peprotech (Rocky Hill, NJ, USA), sulfasalazine, from Sigma (St Louis, MO, USA) and SN-50, from Calbiochem (San Diego, CA, USA).

**Materials and Methods**

**Mouse Lines**

To examine the role of p65 in SGNs, viable p65−/− mice were generated on a Tnfr1−/− background as described previously (Meffert et al., 2003) and maintained as heterozygous. Mice were group-housed in the animal facility of the University of Liege under standard conditions with food and water ad libitum and were maintained on a 12-h light/dark cycle. All animals were taken care of in accordance with the declaration of Helsinki and following the guidelines of the Belgian ministry of agriculture in agreement with EC laboratory animal care and use regulation.

**Primary Cultures of SGNs**

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At the end of the culture period, cells were fixed and immunostained. pups (E18-P10) were lysed on ice using 100 μl of lysis buffer. Peroxidase secondary antibody was described (Lallemend et al., 2005a). Briefly, the number of neurons bearing neuritic processes that were of a defined length of at least three neuronal cell body diameters were counted. Neurites were counted only if they had an obvious attachment to the neuronal soma. The neuritic index was calculated as the ratio of neurons bearing neurites of the defined length or greater per total number of neurons.

**WESTERN BLOT ANALYSIS**

A total of about 20 μg (representing one sample) from rat embryos or pups (E18-P10) were lysed on ice using 100 μl of lysis buffer (1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCL, pH 7.4, 2 mM DTT, 1 mM sodium orthovanadate and protease inhibitors cocktail; Roche). After 30 min, the lysate was cleared of insoluble debris by centrifugation for 10 min at 11,000 g. The supernatant was collected, and the protein concentration was determined using the Bradford method. Protein lysates (30 μg of proteins) were then mixed with an equal volume of gel loading buffer (glyceraldehyde 20%, SDS 4%, Trit 100 mM, β-mercaptoethanol 5% and bromophenol blue) before being boiled for 3 min. After boiling, proteins were loaded onto a 10% SDS-PAGE. The proteins were then transferred on a polyvinylidene difluoride membrane (Amersham, Roosendaal, the Netherland) by semi-dry electroblotting in transfer buffer (Glycine 192 mM, Tris 25 mM and methanol 20%). Blots were then blocked for 1 h at room temperature in blocking buffer [0.2% I-BLOCK (TROPIX, Bedford, MA, USA) diluted in TBS supplemented with 0.05% Tween 20]. The primary antibodies remained at low level during the following days (Figure 1C). mRNA for both p65 and p50 was stable from late embryonic to postnatal stages, mRNA for both p50 and IκB is restricted to the neuronal population (Figures 1A,B). We then analyzed the general expression pattern of p65, p50 and of IκBα in whole protein extracts of SG from E18, E19, P1 and P5 animals. As illustrated in Figure 1C, a semi-quantitative analysis (data are normalized to the values obtained at E18) showed that the level of expression of the three proteins remained unchanged during late embryonic development. However, while the expression of p50 and IκBα was stable from late embryonic to postnatal stages, the expression of p65 protein dramatically decreased at P1 and remained at low level during the following days (Figure 1C).

**RIBOPROBE SYNTHESIS**

pRcCMV plasmids containing anti-sense and sense primers for amplification of p65 and p50 were kindly provided by Dr. S. Memet from Pasteur Institute in Paris. Plasmids were cloned in bacteria mach1TM-T1R (Invitrogen) and isolated using Qiagen miniprep Kit from Qiagen (Germany). The complementary strands for sense and antisense riboprobes were transcribed from either Sp6 or T7 RNA polymerases and labeled using rNTP mix containing digoxigenin-labeled rNTP purchased from Roche Diagnostics (Germany).

**IN SITU HYBRIDIZATION**

Riboprobes were diluted to appropriate concentrations in hybridization buffer (Amresco) containing 50% formamide and were denaturated for 10 min at 65°C. Riboprobes were applied to sections for overnight hybridization in a 50% formamide chamber at 55°C. Sections were washed twice in 0.1 x SSC (300 mM sodium chloride and 30 mM sodium citrate, pH 7.0) at 55°C for 30 min. After a brief wash in Tris buffer (0.1 M Tris-HCL and 0.15 M sodium chloride, pH 7.5), sections were blocked in Tris buffer containing 0.5% blocking reagent (catalog #1096176; Boehringer Mannheim) and 0.3% Triton X-100 and incubated with anti-digoxigenin antibody conjugated to alkaline phosphatase (1:750; Boehringer Mannheim). Sections were exposed to staining solution containing nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate for up to 20 h and viewed using an Olympus AX70 microscope.

**ELECTROMOBILITY SHIFT ASSAY**

Nuclear protein extracts were obtained and processed for electrophoretic mobility shift assay as described previously (Robe et al., 2004).

**STATISTICAL ANALYSIS**

Statistical analyses were performed by one-way ANOVA using a GraphPad Prism program (GraphPad, San Diego, CA, USA). Individual comparisons were performed using Dunnett’s post hoc tests. Data were expressed as the mean ± S.D. and results were considered statistically significant if P < 0.05 for both the one-way ANOVA and the Dunnett’s test.

**RESULTS**

**DEVELOPMENTAL DYNAMIC OF p65, p50 AND IκBα EXPRESSION IN THE SG**

To study the expression of the NFκB complex in the SG, we first analyzed the expression of p65 and p50 transcripts by in situ hybridization. Data revealed that at P5 (similar results from E18 to 1 year old, data not shown), mRNA of both p50 and p65 are restricted to the neuronal population (Figures 1A,B). We then analyzed the general expression pattern of p65, p50 and of IκBα in whole protein extracts of SG from E18, E19, P1 and P5 animals. As illustrated in Figure 1C, a semi-quantitative analysis (data are normalized to the values obtained at E18) showed that the level of expression of the three proteins remained unchanged during late embryonic development. However, while the expression of p50 and IκBα was stable from late embryonic to postnatal stages, the expression of p65 protein dramatically decreased at P1 and remained at low level during the following days (Figure 1C).

**p65 IS SELECTIVELY EXPRESSED IN TYPE II NEURONS IN POSTNATAL AND ADULT COCHLEA**

The marked decrease of p65 expression in whole SG observed during the first postnatal week may indicate a general downregulation of p65 or a selective expression within a specific neuronal population. To address this issue, we examined the temporal and spatial distribution of p65 expression on cochlear sections of different
developmental stages using antibodies against p65, βIII-tubulin (a general neuronal marker; Fanaraga et al., 1999) and peripherin, a type III intermediate filament protein that serves as a reliable marker for type II neurons (Fanaraga et al., 1999) and peripherin, alpha and beta III-tubulin (an adult stage, where the small size of the p65 immunopositive neurons could be visualized in only a few neurons (Figures 2E–G). A further analysis of the immunostaining at the level of the organ of Corti reveals that they are type II neurons (Figure 3B). Later during development, around P11 when hearing starts in rat, the expression of p65 could no longer be seen within the SG (data not shown). It reappeared however, at around P15-16 in type II neuronal cell bodies, but not in their projections, an expression profile that was maintained at adult stage (Figure 2L).

In contrast to p65, the expression of p50 was expressed in all SGNs, but not in their projections, at all stages analyzed (Figures 2M–X).

**TRANSITORY IN VIVO ACTIVITY OF NF-κB DURING EARLY POSTNATAL PERIOD**

The above results suggest the participation of NFκB in biological processes that regulate the early postnatal development of type II neurons. To assess whether NFκB particularly is activated during that period, we studied the DNA-binding activity of p65 in SG at different stages. Using a gel shift assay, we observed that the activity of p65 subunit in SG nuclear extracts was virtually absent at late embryonic stage, appeared at P1 (data not shown) and markedly increased at P5 (Figure 4A, NFκB activity, E19: 1 ± 0.13, P5: 7.27 ± 1.61; n = 4, P < 0.001, t-test, normalized to E19). These results were confirmed by analyzing the subcellular localization of p65 in type II neurons at postnatal stages. Indeed, one hallmark of NFκB activity is its translocation within the nucleus, which could be observed in many type II neurons at P4-5, as illustrated in Figure 4B. Immunolabeling for p65 was never observed in type I neurons after birth.

**BDNF-INDUCED SURVIVAL OF SGNs REQUIRES NF-κB SIGNALING DURING EARLY POSTNATAL PERIOD**

The postnatal period is characterized by a refinement of the inner-oval of the organ of Corti by SGNs, and a concomitant loss of neurons within the type II population, which depends on BDNF for their survival (Barclay et al., 2011). To investigate the implication of NFκB in the signaling of the trophic support mediated by neurotrophins on SGNs during this critical period of development, SGNs from different stages of development, from embryonic (E18 and E19) or postnatal (P1, P2, P4, P6, and P8) animals, were cultured for 24 h in the presence of BDNF or NT3 together with specific inhibitors of the NFκB pathway, i.e., SN50 that blocks nuclear translocation of NFκB (Lin et al., 1995) and sulfasalazine (SZ) that interferes with the phosphorylation of IκB (Wahl et al., 1998). Both neurotrophins were shown to significantly increase survival of SGNs (P < 0.01 at P8 and P < 0.001 at E18-P6, compared to control) from embryonic or postnatal animals, as previously shown (Malgrange et al., 1996; Mou et al., 1997, 1998). Strikingly, while inhibitors of NFκB did not affect the survival-promoting activity of NT3 at all stages analyzed (P > 0.05, Figures 5A,C), the trophic action of BDNF was specifically and only affected during the early postnatal period of development, from P1 to P6, with a maximum of inhibition observed at P4 (Figures 5B,C, P > 0.05, BDNF+SN50 compared...
FIGURE 2 | Distribution of p65 and p50 proteins in the developing cochlea. (A–L) Representative confocal images for the expression of p65 subunit (green) and the neuronal marker TUJ1 (red) on rat SG (A–C, E–G, I–K; scale bar: 100 μm) and corresponding organs of Corti (OC; D, H, L; scale bar: 40 μm) from E19, P5 and P35 rat cochlear sections. Inset in A (A’), represents immunoblotting for p65 from P5 rat SG total protein extract which reveals a specific single band staining at the expected molecular weight. (M–X) Representative confocal images for the expression of p50 subunit (green) and the neuronal marker TUJ1 (red) on rat SG (M–O, Q–R, U–W; scale bar: 100 μm) and OC (P, T, X; scale bar: 40 μm) from E19, P5 and P35 rat cochlear sections. Inset in M (M’), represents immunoblotting for p50 from P5 rat SG total protein extract which reveals a specific single band staining at the expected molecular weight. On the same immunoblot, the specific band corresponding to p105 is not shown.
to control). In addition to their survival action, neurotrophins have been shown to affect axonal growth both in vivo and in vitro (Defourney et al., 2011). Interestingly, inhibitors of NFκB did not change the BDNF-induced axon growth of SGNs from P4 animals (P > 0.05), suggesting a specific requirement of NFκB signaling for survival but not general axon growth promoted by BDNF.

**LOSS OF PERIPHERIN+ SGNs IN THE ABSENCE OF p65 IN VIVO**

To examine the requirement for p65 during development of type II neurons in vivo, we changed the model system and analyzed null mutant mice for p65. Since p65−/− mice die at E14–15 from massive liver apoptosis mediated through TNFR1 signaling (Beg et al., 1995; Rosenfeld et al., 2000), we generated mice that were deficient in both p65 and Tnfr1 and analyzed neuronal phenotype in cochlea of young adult animals (P21). Interestingly, although the total number of SGNs (i.e., βIII-tubulin+ cells per section) showed no significant difference between Tnfr1−/− and Tnfr1−/−; p65−/− mice (Tnfr1−/−; p65+; 92.3 ± 14.4; Tnfr1−/−; p65−/−; 74 ± 4.8, n = 3, P > 0.1), the number of peripherin+ type II neurons (which only represents around 10% of the whole neuronal population) was dramatically reduced in the absence of p65 (Figures 6A–C), whereas experiments with wild-type mice yielded similar numbers as seen for Tnfr1−/−; p65+/+ (data not shown). Together with our in vitro analysis, these data show that BDNF through NFκB signaling is required to support a significant fraction of the type II neuronal population during early postnatal development of the cochlea.

We next sought to define the dynamic of BDNF expression during this critical postnatal period of development. At P0, BDNF was found to be expressed in the sensory epithelium and nerve endings below the hair cells while the cell bodies of the neurons were not or barely positive (Figure 6D,E), regardless of the type (in both peripherin positive and negative SGNs, data not shown), it was absent in hair cells (Figure 6F), confirming previous results (Wiechers et al., 1999) and suggesting a local (autocrine and/or paracrine) function of BDNF in sustaining survival of type II population during the postnatal period of development.

**DISCUSSION**

Although the assignment of NT3 for the survival of type I SGNs during development is still unclear, the trophic function of BDNF on type II neurons has been largely documented, both in vitro and in vivo (Ernfors et al., 1995; Wiechers et al., 1999; Schimmang et al., 2003; Barclay et al., 2011). However, BDNF is expressed in both IHCs and OHCs during early development and its high affinity receptor TrkB, in all SGNs, raising the question to know what
We show here that the expression of p65 is present in virtually all neurons until birth and selectively expressed at high cellular levels for instance by regulating either the expression of neuron type-specific miRNA expression or the relative amount of p50 and p65 in the neuron, what would impact the formation of potentially transcriptionally active p65/p50 heterodimers versus p50 homodimers that are transcriptionally inactive or negative regulators of transcription (Hayden and Ghosh, 2012). This reorganization is accompanied by a 25% neuronal loss that mostly concerns the type II population (Rueda et al., 1987; Barclay et al., 2011) and likely participates in the elimination of type II afferents underneath the IHCs. Interestingly, early postnatal type II neurons are rescued by BDNF in vivo (Barclay et al., 2011). NFκB signaling has been shown to act downstream of neurotrophic factor receptors (including Trks) activation to promote neuronal survival (Magirwar et al., 1998; Huang and Reichardt, 2011). NFκB signaling has been shown to act downstream of neurotrophic factor receptors (including Trks) activation to promote neuronal survival (Magirwar et al., 1998; Huang and Reichardt, 2011). The observation in our study of a specific requirement of p65 signaling for BDNF-induced survival of early postnatal SGNs in vitro and of its specific role in regulating survival of type II neurons in vivo strongly suggest that NFκB represents a key molecular component driving the differential survival promoting action of BDNF on type II neurons. The maintenance of p65 expression in type II SGNs in adult raises the question whether NFκB would play a similar survival function in vivo.

![Figure 5](image-url)

**FIGURE 5** NFκB is necessary for BDNF but not NT3-induced survival of postnatal SGNs. (A–C) SGNs were cultured for 24 h with BDNF or NT3 (each at 20 ng/ml) and in the presence of specific inhibitors of the NFκB signaling. SN50 (5 μM) or sulfasalazine (1 mM, sz; P4, C). Note the absence of effect of the inhibitors on survival function of NT3 while they dramatically decrease the survival promoting action of BDNF (n = 3–8; *P < 0.05, **P < 0.01, ***P < 0.001, compared to neurotrophin alone).
on this population under stress conditions, where neuroinflammatory factors and calcium signaling, both known to be able to activate NFκB (Meffert et al., 2003; Hayden and Ghosh, 2012), play an important role (Gale et al., 2004; Lallemend et al., 2005b; Toonabene et al., 2006; Keithley et al., 2008).

The spatio-temporal dynamic expression of p65 in SGNs observed in our study is accompanied by changes in the expression profile of BDNF in the cochlear system. Indeed, we and others have shown that BDNF is downregulated in the sensory epithelium and start to be expressed in the majority of neurons (type I and II) during the first postnatal week (Wheeler et al., 1994; Wiechers et al., 1999). This downregulation of BDNF in the organ of Corti might contribute to afferent fiber retraction from the hair cells (Wheeler et al., 1994). The initiation of BDNF expression in SGNs at the same stage could then provide a transient autocrine and/or paracrine signal whereby type II neurons, deprived of organ of Corti-derived trophic signals, could survive until BDNF is again supplied by their target at later stage (Wiechers et al., 1999; present study). The expression of p65 in SGNs is also regulated during the development of the peripheral nervous system.

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AUTHOR CONTRIBUTIONS

François Lallemend, Brigitte Malgrange, and Saïda Hadjab designed and supervised the study; Renaud Vandenbosch, Eva Chocholova, Pierre A. Robe, Yiqiao Wang, Cécile Lambert, François Lallemend, and Saïda Hadjab performed experiments; Renaud Vandenbosch, Eva Chocholova, Pierre A. Robe, Yiqiao Wang, Cécile Lambert, François Lallemend, Brigitte Malgrange, and Saïda Hadjab analyzed data; Renaud Vandenbosch, Gustave Moosnen, François Lallemend, Brigitte Malgrange, and Saïda Hadjab wrote or revised the manuscript, with input from co-authors;
François Lallemend, Brigitte Malgrange, and Saida Hadjab are co-author seniors.

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