Kcnip1 a Ca\(^{2+}\)-dependent transcriptional repressor regulates the size of the neural plate in Xenopus

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A B S T R A C T

In amphibian embryos, our previous work has demonstrated that calcium transients occurring in the dorsal ectoderm at the onset of gastrulation are necessary and sufficient to engage the ectodermal cells into a neural fate by inducing neural specific genes. Some of these genes are direct targets of calcium. Here we search for a direct transcriptional mechanism by which calcium signals are acting. The only known mechanism responsible for a direct action of calcium on gene transcription involves an EF-hand Ca\(^{2+}\) binding protein which belongs to a group of four proteins (Kcnip1 to 4). Kcnip protein can act in a Ca\(^{2+}\)-dependent manner as a transcriptional repressor by binding to a specific DNA sequence, the Downstream Regulatory Element (DRE) site. In Xenopus, among the four kcnips, we show that only kcnip1 is timely and spatially present in the presumptive neural territories and is able to bind DRE sites in a Ca\(^{2+}\)-dependent manner. The loss of function of kcnip1 results in the expansion of the neural plate through an increased proliferation of neural progenitors. Later on, this leads to an impairment in the development of anterior neural structures. We propose that, in the embryo, at the onset of neurogenesis Kcnip1 is the Ca\(^{2+}\)-dependent transcriptional repressor that controls the size of the neural plate.

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1. Introduction

Calcium (Ca\(^{2+}\)) signaling plays an essential role throughout vertebrate development, from fertilization to organogenesis. In particular, Ca\(^{2+}\) fluxes have been implicated in dorsal–ventral axis establishment, convergence extension movements [1,2] and in neuronal differentiation during neural tube closure [3,4]. Furthermore, Ca\(^{2+}\) is required for the specification of the vertebrae embryonic nervous system during neural induction [5–7].

Neural induction occurs in the dorsal ectoderm at the onset of gastrulation. This process is controlled by morphogens secreted by the dorsal mesoderm like Noggin, Chordin, Follistatin, Xnr3 and Cerberus, which antagonize bone morphogenetic protein (BMP) signaling [8]. We have previously demonstrated that neural induction is associated with transient increases in intracellular Ca\(^{2+}\), resulting from the activation of L-type Ca\(^{2+}\) channels. These intracellular Ca\(^{2+}\) increases are necessary and sufficient to engage the ectodermal cells into a neural fate, inducing proneural genes such as zic3 [5,9].

Through a differential screening between naïve and Ca\(^{2+}\)-stimulated ectoderms we have isolated genes encoding transcriptional regulators and RNA binding proteins downstream of the Ca\(^{2+}\) signaling pathway [10–13]. Among the isolated genes we demonstrated that the arginine methyltransferase pmnt1b acts as an early proneural gene that regulates zic3 transcription. Interestingly, pmnt1b expression is a direct Ca\(^{2+}\) target that does not require de novo protein synthesis [11].

Actually the only known direct Ca\(^{2+}\)-dependent transcriptional regulation occurs via the Ca\(^{2+}\) sensor DREAM (Downstream Regulatory Element Antagonist Modulator). The DREAM protein, also called calsenilin, KChIP-3 or Kcnip3, is an EF-hand Ca\(^{2+}\) binding protein which belongs to a group of four proteins that regulate the membrane expression and the gating of Kv4 potassium channels [14]. Interestingly, this protein is also able to bind to specific DNA sequence; the DRE (Downstream Regulatory Element) site. The affinity of DREAM to the DRE is modulated by the Ca\(^{2+}\) occupancy of its EF-hand sites. DREAM acts in a Ca\(^{2+}\)-dependent manner as a transcriptional repressor [15,16] or activator [17,18].

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To simplify, hereafter we will use the kcnip acronym to refer this family of Ca\(^{2+}\) sensors. In mammals, the four Kcnips (Kcnip-1 to 4) are predominantly expressed in specific neuronal regions of adult brain in rodent and human [19–21]. They are also found in nonneuronal tissues including, the thyroid gland [22,23], the hematopoietic progenitor cells [24] and the T and B lymphocytes [25,26].

There is no data concerning Kcnip expression during neural induction in vertebrates. In the mouse, kcnip3 transcript is detected at E10.5 [27] which occurs 3 days after neural induction and kcnip1, 2 and 4 are not detected before E13 [19]. In the fish Danio rerio, the embryonic expressions of kcnip1b and 3 are detectable not before somitogenesis [28].

In this article we ask if in the amphibian Xenopus laevis, Kcnip family members are important transcriptional regulators during neural induction. We have determined that only kcnip1 is timely and spatially present in the presumptive neural territories. The loss of function of kcnip1 impairs the development of anterior neural structures, which reveals a functional involvement of the Kcnip family member in early neurogenesis. In addition, we have shown that Xenopus Kcnip1 is able to bind DRE sites in a Ca\(^{2+}\)-dependent manner and to control the proliferation of neural progenitors. We propose that Kcnip1 is a Ca\(^{2+}\)-dependent transcriptional repressor that participates in the regulation of the neural plate expansion at the origin of the nervous system in Xenopus embryo.

2. Material and methods

2.1. Embryos

X. laevis embryos were obtained and staged using standard procedures [11,29]. Embryos were raised in 0.1X Normal Amphibian Medium.

2.2. Constructs, mRNA, morpholino microinjections

Plasmids containing the four full length clones for X. laevis kcnips (kcnip1 to 4) were obtained from different libraries (Table S2). Kcnip1 was PCR-introduced into pCS2 or in frame with GFP into pCS2-GFP vectors (Forward 5′-CCGATCCATGAGGCACTGATGGA-3′; Reverse 5′-CCGATGGCACTGATG-3′), at BamHI and EcoRI restriction sites, underlined, with or without stop codon, in bold). For in vivo experiments, mRNAs were synthesized using the mMESSAGE mMACHINE kit (Ambion) from NotI-linearized plasmids. For microinjections 6 ng of kcnip1-GFP were injected per blastomere. The anti-sense morpholino was PCR-introduced into pCS2 or in frame with GFP into pCS2-GFP vectors (Forward 5′-CGGATCCATGAGGCACTGATGGA-3′; Reverse 5′-CCGATGGCACTGATG-3′), at BamHI and EcoRI restriction sites, underlined, with or without stop codon, in bold). In vivo experiments, mRNAs were synthesized using the mMESSAGE mMACHINE kit (Ambion) from NotI-linearized plasmids. For microinjections 6 ng of kcnip1-GFP were injected per blastomere. The anti-sense morpholino was PCR-introduced into pCS2 or in frame with GFP into pCS2-GFP vectors (Forward 5′-CCGATCCATGAGGCACTGATGGA-3′; Reverse 5′-CCGATGGCACTGATG-3′), at BamHI and EcoRI restriction sites, underlined, with or without stop codon, in bold).

2.3. RT-PCR, in situ hybridization, immunohistochemistry

RT-PCR analysis was performed on RNA extracts from staged embryos as described [12]. For the separation of the outer and inner layers, ectoderm explants were isolated from stage 10 embryos and transferred to Ca\(^{2+}\) and Mg\(^{2+}\)-free medium [30]. This resulted in the disaggregation of the inner layer cells, whereas the outer layer cells remained intact, facilitating the separation between the two layers. Primer pairs are presented in Table S3. Whole-mount ISH was carried out according to [31]. For kcnip1 anti-sense and sense digoxigenin-labeled probes were respectively synthesized by T7 and SP6 RNA polymerases (Promega), with the full length cDNA kcnip1 used as template. The other plasmids used as templates were prmt1b, zic3, sox2, sox8, n-tubulin, pax6, p27x1c1, k81 and myod (Table S4). Stained embryos were then embedded in 3% low melting agarose for vibratome sectioning. For immunohistochemistry analysis, in vivo expression of GFP signals was performed on embryos fixed at stage 9 or stage 14 [13]. The proliferative cells were stained with anti-phospho-Histone 3 (Ser10) anti-body (1/1000, Up-state) and revealed using an Alexa-555-coupled secondary anti-body (Fisher Scientific). Nuclei were counterstained with To-Pro3 (1/1000, Molecular Probes). Fluorescent images were obtained using confocal facilities (Leica SP2).

3. Results

A DRE-binding activity is present at blastula and neurula stages in Xenopus embryos

The four Kcnip members are capable of binding DNA at DRE sites in a Ca\(^{2+}\) dependent manner [16] and make the direct link between Ca\(^{2+}\) signaling into transcriptional activation. To test this, we performed electrophoretic mobility shift analysis (EMSA) using nuclear extracts from embryos at blastula (stage 9) or at early neurula (stage 14) with double-stranded oligonucleotide encompassing the mouse NCX3-DRE site [35]. DRE-retarded bands were observed in stage 9 and stage 14 embryos extracts with the NCX3-DRE consensus probe (Fig. 1A, lanes 2 and 6 respectively). The retarded bands were competed with 20-fold excess of a cold NCX3-DRE probe (Fig. 1A, lanes 3 and 7) and affected by the mutated-DRE site (Fig. 1A, lanes 5 and 8 respectively). Furthermore for stage 9 nuclear extract, this binding was significantly decreased in the presence of 10 μM CaCl\(_2\) (Fig. 1A, lane 4), as initially established in the case of mammalian KCNIP3 [15]. These data suggest that blastula and neurula stage X. laevis embryos contain a Ca\(^{2+}\)-dependent DRE-binding activity that corresponds to DRE-binding Kcnip specificity. In addition, in silico analysis reveals potential DRE sites in the proximal 5′UTR of prmt1 gene from X. laevis (accession number BC549595) in sense orientation at −40 nt position and anti-sense orientation at −6 nt position from the AUG codon (Fig. 1B). A putative DRE binding site was also found in the anti-sense orientation at the most proximal position (−5 nt) of prmt1 gene from Xenopus tropicalis (accession number NM_001005629). Furthermore two important genes involved in the control of neural progenitors proliferation, foxm1 and sox2, also present
potential DRE sites in their regulatory region (Fig. 1B). Indeed, DRE site in the close proximal position is found in foxm1 gene from X. laevis (AJ853462) in sense orientation at −5 nt from first codon and from X. tropicalis (XM_004912735) in anti-sense orientation at −3 nt. A second anti-sense DRE site is present upstream in X. tropicalis foxm1 at −80 nt. Sox2 gene exhibits potential DRE sequence in relative more distal positions, −220 nt and −157 nt in X. laevis (NM_001088222), and −236 nt, −175 nt and −50 nt in X. tropicalis (NM_213704).

3.1. Identification of Kcnip family in X. laevis

In order to characterize the molecular element responsible for the DRE binding activity, we screened available databases to find the X. laevis Kcnips by homology with mammalian sequences. In mammals, the four kcnip family genes encode several protein isoforms, which differ in their N-termini depending on the alternative promoter and splicing use [19] whereas the C-termini are more identical and contain the dimerization domain, the EF-hands Ca²⁺ binding sites motifs and the residues involved in DNA sequence recognition [36]. Predicted sequences concerning X. laevis were extracted from Xenbase and NCBI libraries. The putative deduced Kcnip members of X. laevis were aligned with the human and mouse protein orthologs (Fig. S1). The entire C terminal sequences that comprise the 4 EF-hands (boxes in gray) are well conserved, and sequence variations mainly concern the N terminus domain. We were able to attribute each sequence issued from X. laevis databases to the nearest isoform determined by the alignment with mammalian sequences, and to confirm the annotation we here adopted for Xenopus Kcnips according to the nomenclature (http://www.xenbase.org). Full length cDNA BC074264 supports the Kcnip1 isoform encoded by kcnip1-Ib variant (Fig. S1A), and full length cDNA BC097806 displays highest homology to kcnip4-Ie variant (Fig. S1D). A partial sequence for kcnip3 variant was found in databases (DC056397) and corresponds to kcnip3-Ib variant encoding a type 2 isoform (Fig. S1C). Kcnip2 identification from Xenbase is contained by the cDNA referred to BC082465 (Fig. S1B). To summarize these alignments, the highest similarities in amino acids restricted to ORFs are given in Table S1 and only the closest isoform of each respective member in the Kcnip family is considered here. According to a high degree of homology, even with the exception of Kcnip2 bearing only 71% identities to mammals, all these X. laevis predictive proteins belong to the Kcnip family (Table S1).

A phylogenetic tree was constructed with the Phylip Neighbor-joining method (Fig. S2), where we introduced the predictive Kcnip isoforms isolated from amphibians, zebrafish and chicken, with human and mouse proteins. The first appearance of Kcnips in evolution concerns the insect phylum, with a unique Kcnip related protein in Drosophila melanogaster harboring about 40% identity with the mammals [37]. X. laevis Kcnip1, 3 and 4 are indeed related to their respective subgroup except for X. laevis Kcnip2 which shows proximity with Kcnip1 sequence. For synteny analysis, we examined from databases the genomic regions containing Kcnips. The close neighbor genes flanking Kcnip orthologs are mainly conserved between human, mouse, chicken, X. tropicalis and X. laevis genomes, despite less information concerning the last species (Fig. S3). The 3′ flanking region for Xenopus Kcnip1 differs from those for human, mouse or chicken downstream to Tlk3. X. tropicalis kcnip2 region is more closely related to Gallus KCNIP2. The proposed position of kcnip2 in X. laevis genome is misleading, confirming the ambiguity for this gene in this species. Surprisingly no KCNIP3 gene is found in chicken. The 5′ flanking genes upstream to
3.2. Temporal expression of kcnip1 during Xenopus development

To investigate the temporal expression pattern of the X. laevis kcnip members, specific primers were chosen for the putative variants according to Table 1. RT-PCR on RNAs extracted from X. laevis embryos are shown in Fig. 1C. The Kcnip1 homolog is expressed in all stages throughout embryonic development, from fertilized egg (stage 1) to tadpole stages (stage 46). The transcripts for kcnip3 and kcnip2 are expressed far after neural induction, at stage 21 and stage 25 respectively. Kcnip4 is not amplified before stage 46. Since the temporal expression pattern of kcnip1 is compatible with a role in neural induction in X. laevis, we focused on kcnip1 for further functional analysis.

3.3. Spatial expression pattern of kcnip1 revealed strong expression in neural territories

The spatial expression pattern of kcnip1 was analyzed by whole mount in situ hybridization (ISH). Maternal mRNA is localized in the animal blastomeres during the cleavage stages (Fig. 2A1, A2). After the zygotic transcriptional switch at midblastula, kcnip1 is expressed at the onset of gastrula throughout the animal pole; in the ectoderm and mesoderm (Fig. 2B–C). To better investigate kcnip1 expression during gastrulation we performed ISH on sections of gastrula embryos (stage 10.5). As shown in Fig. 2C, the expression of kcnip1 can be detected in both the ventral and dorsal ectoderm and mesoderm; no staining was detected in the endoderm. To further analyze kcnip1 expression in the ectoderm, we have isolated the superficial outer layer (nonneuronal epithelial layer) from the deep inner layer (the sensorial neuroectoderm layer) of isolated ectoderm excised at early gastrula (stage 10) and analyzed in both layers kcnip1 mRNA expression by RT-PCR. Kcnip1 mRNA is found in both the outer and the inner layers (Fig. S4). During neural tube closure stage (stage 18), kcnip1 is highly expressed in the neural folds (Fig. 2D) where a strong signal was observed in the most anterior part from which the entire brain will develop. Central transverse sections of sibling stage clearly highlight the expression pattern in the neuroepithelium tissue and in the somitic mesoderm (Fig. 2E). Notochord was faintly marked.

During organogenesis, kcnip1 is expressed at a high level in neural structures (stage 33/34, Fig. 2F). Indeed, all the developing central nervous system (CNS) was strongly stained, from the anterior to the posterior, namely the telencephalon, the mesencephalon, the rhombencephalon, and the spinal chord (Fig. 2F and insert). Sensory neurogenic placodes, including the olfactory gland, the ocular vesicle, the otic vesicle, the cranial ganglia and the branchial arches which originate from the neural crest, also expressed a high level of kcnip1 mRNA. Mesodermal expression is lower compared with the neural one, restricted to the inter somitic structures, and to the pronephros. No staining was seen in the cement gland and in the endodermal derivatives. Transverse sections at the head and trunk levels confirm the specific expression of kcnip1 in the CNS, highly present in the diencephalon and neuroretina (Fig. 2F1) and in the spinal chord (Fig. 2F2). The kcnip1 transcript exhibited a low but significant expression in the pronephric tubule and its expression disappeared from the notochord (Fig. 2F2). The robust spatial and temporal kcnip1 expressions during the early development in Xenopus embryos suggest a function for kcnip1 during early neurogenesis.

Table 1

Quantification of the anterior expression domain expansion of prmt1b in MoK1 and MoC-injected embryos.

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Mean are given ± mean deviation. arb.units: arbitrary units.

* This value corresponds to the width of the anterior expression domain of prmt1b on the MoK1-injected side.

† This value corresponds to the width of the uninjected side, as shown in Fig. 2A.
Fig. 2. Kcnip1 mRNA distribution during Xenopus embryonic development. (A) Maternal expression at stage 6.5. (A1) Animal pole view showing expression in the micromeres. (A2) Lateral view; the macromeres do not express kcnip1. (AP) Animal pole. (VP) Vegetal pole. (B) At gastrula stage (stage 10.5) kcnip1 is expressed in the dorsal ectoderm (ect). Dorsal to the top, dashed line and arrow: blastoporal lip (bp). (C) In the corresponding gastrula sagittal section, kcnip1 mRNA is expressed in ectoderm (ect) and mesoderm (mes); and endoderm (endo) is not labeled. Blastoporal lip is indicated by arrow. (D) Anterior view of a late neurula, kcnip1 is expressed in the whole neural plate. (E) Transverse section of (D) showing a high expression of kcnip1 in the neural groove (ng), the closing neural tube (cnt) and somitic mesoderm (sm). Arch, archenteron, n, notochord. Bar for A, B, C, D, E 0.5 mm. (F) Lateral view of stage 33/34 larvae and detail of the anterior part. Kcnip1 is strongly expressed in the different structures of the central nervous system (forebrain, midbrain, rhombomeres), in the spinal chord (sp) in the eyes (e), also in branchial arches (ba), in intersomitic structures (s) and in pronephric tubules (pt). Kcnip1 is not expressed in the cement gland (cg). Bar 1 mm. F1 and F2 transverse sections at the indicated levels visualize kcnip1 expression in the diencephalon (Di), in the spinal chord and pronephric tubules, and n notochord. Bar F1 and F2: 0.2 mm.
the neural contribution of Kcnip1 is invalidated without affecting any mesodermal activity [39]. Suppression of Kcnip1 by MoK1 resulted in a significant increase in prmt1b (79%, n = 29) and of its downstream target zic3 (88%, n = 35) in the anterior neural plate at stage 16, as shown by ISH (Fig. 4). By contrast, MoC had no effect on prmt1b or zic3 expression (Fig. 4). This enlargement was quantified by the ratio of the width of the anterior expression domain of prmt1b on the MoK1-injected side relative to the uninjected side of neurula stage embryos (Fig. 4 and Table 1). Injection of MoK1 into one dorsal blastomere of an 8-cell embryo caused a 30% (n = 10) increase of prmt1b expression domain compared with MoC-injected embryos. Kcnip1, when bound to DRE sites is a transcriptional repressor. Therefore, the reduction of Kcnip1 protein by morpholino injection leads to a diminished transcriptional repression. As a direct consequence, this induces the increase in the transcription of its target genes such as prmt1b.

Sox2 is a member of SoxB1 subgroup of transcription factors known to control neural progenitor proliferation [40]. Like for prmt1b and zic3, injection of MoK1 induced a significant increase in sox2 mRNA (85%, n = 20). This expansion of the neural plate is accompanied by a reduction of the neural crest cells marker sox8 expression (88%, n = 17) and by a lateral shift of the epidermal marker k81 (91%, n = 11). No apparent change was observed for the expression pattern of earliest muscle-specific marker myod. This result demonstrates clearly that the increase

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**Fig. 3.** Localization of Kcnip1, effect of morpholino on kcnip1 translation and DRE binding activity. (A) Confocal imaging of double labeled ectoderm cell dissected at stage 9 over expressing Kcnip1-GFP (in green, left panel), and the nucleic marker ToPro3 (in red, middle panel). The merge imaging shows a co-localization of these two probes (merge, right panel). Arrows point nuclei. (B) Morpholino MoK1 blocks Kcnip1-GFP translation. Morpholino efficiency was verified by Western blot with an antibody against GFP on stage 13 lysates from whole embryos injected with kcnip1-GFP. Anti-α-tubulin attests equivalent loading. Control corresponds to uninjected embryo extract (lanes 1, 4, 7). Embryos were injected at stage 2 with 6 ng of control morpholino (MoC, lanes 2, 3) or with 6 ng of morpholino against Kcnip1 (MoK1, lanes 5, 6), and with 50 pg of GFP mRNA (GFP, lanes 3, 6) or with 6 ng of kcnip1-GFP mRNA (Kcnip1-GFP, lanes 2, 5) respectively. The GFP protein is expressed wherever the morpholino used, whereas MoK1 (lane 5) but not MoC (lane 3) inhibits Kcnip1-GFP translation. (C) Morpholino MoK1 reduces DRE binding activity. The DRE probe was submitted to EMSA after incubation with Xenopus nuclear extracts from MoC injected embryo at stage 9 (lane 1, MoC) or stage 14 (lane 4, MoC), or from MoK1 injected at stage 9 (lane 2, MoK1) or stage 14 (lane 5, MoK1), or from morpholino targeting 5′ UTR kcnip1 (MoK1a) at stage 9 (lane 3, MoK1a) or stage 14 (lane 6, MoK1a) demonstrating that MoK1 significantly inhibits the DRE binding activity due to Kcnip1.

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**Fig. 4.** Kcnip1 invalidation impairs neural progenitor fate. Kcnip1 invalidation was performed by injection of MoK1 into D1 blastomere at stage 3. MoK1 induces neural plate enlargement at neurula stage, on injected side (right panels), with a significant percentage for the extension of the expression pattern of proneural genes, prmt1b (79%, n = 23/29), zic3 (88%, n = 31/35), sox2 (85%, n = 17/20). Conversely, the neural crest marker sox8 is reduced (88%, n = 15/17), the epidermal gene k81 is laterally shifted (91%, n = 10/11) and the early muscle specific marker myod is unaffected (80%, n = 4/5). Control morpholino MoC has no effect (upper panels). The enlargement relative to the expression domain prmt1b of the MoK1-injected side (30%, n = 10) was measured as the ratio of a/b. The same measurement was done for MoC injected embryos (see details in text). Injected side on the left (red gal), bar scale: 0.5 mm.
3.6. Kcnip1 knockdown results in increased proliferation of neural progenitors at neurula stage

We next asked whether kcnip1 loss-of-function expands the neural plate by increasing the cell proliferation of neural progenitors. To address this, we analyzed the mitotic cells in the neural plate of stage 14 embryos by immunostaining with anti-phosphorylated histone H3 (pH3) [41]. MoK1 or MoC were injected with GFP mRNA as a tracer into one dorsal blastomere of 8-cell embryos and the number of pH3 positive cells counted (Fig. 5A). MoK1 caused a 1.6-fold increase in cell proliferation over the control side (Fig. 5B, n = 7, p = 0.005). There was no difference between the MoC and the uninjected side of the control embryos (n = 3, p = 0.177, Fig. S3).

To further confirm that Kcnip1 affects proliferation, we examined whether it can alter the expression of two regulators of cell division. Foxm1, a forkhead transcription factor required for cell proliferation of neural precursors in Xenopus neuroectoderm [42] and p27xic1, a cyclin-dependent kinase inhibitor, which is expressed in post mitotic cells in the neural plate [43]. Embryos injected with MoK1 showed an increase of foxm1 expression (85%, n = 7) and a reduction of p27xic1 expression (81%, n = 32) in the anterior neural plate on the injected side (Fig. 5C). Altogether these data indicate that the early suppression of kcnip1 provokes an enrichment of neural progenitors through the stimulation of cell proliferation.

3.7. Loss of Kcnip1 results in the inhibition of neural differentiation and a reduction of anterior neural structures and eye formation

In Xenopus neural plate, the over-expression of sox2 expands the neural progenitor cell population and prevents their differentiation [40,44,45]. Since we showed here that kcnip1 controls the expression of sox2, we wanted to determine the effect of kcnip1 suppression on neuronal differentiation. The expression of the neuronal marker n-tubulin (n-tub) was analyzed by ISH at the neural tube closure (stage 20). The anterior domain of n-tub expression pattern was down-regulated in kcnip1 morphants while the most posterior domain is unaffected (88%, n = 25, Fig. 6A). To further analyze the effect of kcnip1 loss-of-function on the anterior neural development, we performed ISH for pax6, a homeobox transcription factor. At gastrulation, the anterior expression domain of pax6 marks the eye field [46]. Injection of MoK1 in one dorsal blastomere of 8-cell embryos results in the reduction of pax6 expression in the eye field at neurula (86%, n = 15, Fig. 6A) when compared with MoC injected embryos, suggesting that the formation of the eye field was impaired. This leads to a strong reduction of the eye morphology at tailbud stage (stage 33/34, Fig. 6B) and swimming larva (stage 46) 82% (n = 25) display micro-or anophthalmia (Fig. 6C). All of these results are further confirmed by transverse sections at stage 33/34. Anterior sections revealed that kcnip1 loss-of-function results in a severe distortion and reduction of the diencephalon and affects eye morphology as seen by the reduction of the pigment layer and of the neuroretina (Fig. 6 C1). More posterior cross-sections passing through otic vesicle showed the impairment of this structure on the injected side and a reduction of the rhombencephalon, whereas the notochord is not affected (Fig. 6 C2). More caudally, the symmetry of the embryo is not affected by the suppression of kcnip1 as shown by the position and the structure of the spinal chord, the notochord and the presence of pronephric tubules (Fig. 6 C3).

Taken together, these data indicate that the suppression of kcnip1 results in n-tubulin and pax6 down-regulation in anterior neural domain leading to the impairment of anterior neural development.

4. Discussion

Altogether our data indicate that the neuronal Ca\(^{2+}\) sensor Kcnip1 acts, at the onset of neurogenesis, as a transcriptional repressor to
regulate neural progenitor proliferation in order to control the size of the neural plate.

In *Xenopus* embryos we show that all four *kcnip* family members are expressed but with distinct temporal expression patterns. *kcnip1* is expressed throughout embryonic development and is the only member that is expressed during gastrulation. ISH analysis confirms that the ectoderm expression of *kcnip1* starts before the onset of neural induction and is restricted in the neural territories at later stages. In contrast, in zebrafish and mouse, the expression pattern of *kcnip* members are revealed when brain structures are already formed [19,27].

Among the *kcnip* proteins, *kcnip3* has been shown to act in the nucleus as Ca\(^{2+}\)-dependent transcriptional regulator [15,18]. However, other *kcnip* proteins are able to bind to DRE sites and to act as Ca\(^{2+}\)-dependent transcriptional regulators of genes whose promoters contain DRE sites, including circadian genes [16], cytokine genes such as IL2, 4 and IFN\(\gamma\) in T lymphocytes [25] and in the thyroid cells *pax8* and *fox1E* [22]. In this study, in the amphibian *X. laevis* embryos during early neurogenesis, we demonstrate that *Kcnip1* possesses a Ca\(^{2+}\)-dependent DRE-binding activity.

According to the cellular models, *Kcnip* proteins have been shown to be positively or negatively affect cell proliferation by controlling cell-cycle regulators. In murine thyroid cells proliferation and IFN\(\gamma\) dependent transcriptional regulators of genes whose promoters contain DRE sites, including circadian genes [16], cytokine genes such as IL2, 4 and IFN\(\gamma\) in T lymphocytes [25] and in the thyroid cells *pax8* and *fox1E* [22]. In this study, in the amphibian *X. laevis* embryos during early neurogenesis, we demonstrate that *Kcnip1* possesses a Ca\(^{2+}\)-dependent DRE-binding activity.

During gastrulation and neurulation the neuroectoderm cells proliferate but at a relatively low rate. All neural progenitors divide once during this period [47]. Our data show that the loss-of-function of *kcnip1* positively controls proliferation by increasing *foxl1* and reducing the cyclin-dependent kinase inhibitor *p27xic1*.

Before gastrulation the ectodermal cells are multipotent and can give rise either to epidermis or neural tissue. The mechanisms that govern the choice of neural fate require the inhibition of BMP pathway, the activation of the RGF and Ca\(^{2+}\) signaling pathways [48]. ISH analysis shows that *kcnip1* is expressed in the dorsal and in the ventral ectoderm at early gastrula. How does *kcnip1* control the size of the neural plate? Since we show that the DNA-binding activity of *Xenopus Kcnip1* is Ca\(^{2+}\)-dependent, we propose that in the dorsal ectoderm the Ca\(^{2+}\) transients are the signals able to release the *Kcnip1*-dependent repression of the neural genes *prmt1* and *sox2* while in the ventral ectoderm, where no Ca\(^{2+}\) transients occurs [5,49], *Kcnip1* protein stays bound to DRE sequences and maintains the repression of its target genes. This mechanism is further supported by the presence of potential DRE-binding sites in the regulatory regions of *prmt1* and *sox2* genes.

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