Targeting the RHOA pathway improves learning and memory in adult Kctd13 and 16p11.2 deletion mouse models

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Research

Abstract

Background

Gene copy number variants have an important role in the appearance of neurodevelopmental disorders. Particularly, the deletion of the 16p11.2 locus is associated with autism spectrum disorder, intellectual disability, and several other features. Earlier studies highlighted the implication of *Kctd13* genetic imbalance in the 16p11.2 deletion through the regulation of the RHOA pathway.

Methods

Here, we generated a new mouse models with a small deletion of two key exons in *Kctd13.* Then we targeted the RHOA pathway to rescue the cognitive phenotypes of the Kctd13 and 16p11.2 deletion mouse models in a pure genetic background. We used a chronic administration of fasudil (HA1077), an inhibitor of the Rho-associated protein kinase (ROCK), for several days, in mouse models carrying a heterozygous inactivation of *Kctd13*, or the deletion of the entire 16p11.2 BP4-BP5 homologous region.

Results

We found that the small *Kctd13* heterozygous deletion induced similar cognitive phenotype to the whole deletion of the 16p11.2 homologous region, in the Del/+ mice. Then we showed the chronic fasudil treatment can restore object recognition memory in adult heterozygous mutant mice for *Kctd13* and for 16p11.2 deletion. In addition, the learning and memory improvement was parallel to change in the RHOA pathway.

Limitations:

The *Kcdt13* mutant line does not recapitulate all the phenotypes found in the 16p11.2 Del/+ model. In particular the locomotor activity was not altered at 12 and 18 weeks of age and the object location memory was not defective in 18 weeks old mutants. Similarly, the increased in locomotor activity was not modified by the treatment in the 16p11.2 Del/+ mouse model suggesting other loci involved in such defects. Then, the rescue was only observed after four weeks of treatment but no long term experiment has been done so far. Finally we did not check the social behaviour that require to work in another hybrid genetic background.

Conclusion

These findings confirm KCTD13 as one target gene causing cognitive deficits in 16p11.2 deletion patients, and the relevance of the RHOA pathway as a therapeutic path for the 16p11.2 deletion.

Nevertheless, they reinforce the contribution of other gene(s) involved in cognitive defects found in the 16p11.2 models in older mice.

Highlights

- Kctd13 haploinsufficiency recapitulates most of the behaviour phenotypes found in the 16p11.2 Del/+ models in a pure genetic background
- Fasudil treatment restores Kctd13 and 16p11.2 Del/+ mutant phenotypes for novel location and novel object recognition memory.
- Fasudil treatment restores the RhoA pathway in Kctd13$^{+/-}$ and 16p11.2 Del/+ models

Introduction

Genetic copy number variants of the 16p11.2 locus are an important risk factor for multiple neurodevelopmental disorders [1–5]. The most recurrent 16p11.2 rearrangements, deletion and reciprocal duplication, induce intellectual disability [6] and Autism Spectrum Disorders [7–12]. In addition, they are associated with other neuropsychiatric disorders, such as epilepsy [11], attention deficit / hyperactivity disorder [13], schizophrenia and bipolar trouble [14]. Body mass index phenotypes and abnormal head size have also been reported in these 16p11.2 rearrangements [11–13, 15, 16]. The most frequent variation of the 16p11.2 region corresponds to change in the genetic interval between *SULT1A1 and SPN1*, encompassing 600 kb and 32 genes. Nevertheless, a study conducted in 2011 found a microdeletion of the 118 kb region, containing MVP, SEZ6L2, CDIPT, ASPHD1 and KCTD13, inside the 16p11.2 genetic interval, segregating with Autism Spectrum Disorder in a family over three generations [17]. Thus, this *MVP-KCTD13* subregion should have a potential key role in the neuropsychiatric features linked to 16p11.2 rearrangement.

The modelling of the 16p11.2 rearrangements through animal models recapitulates the human genetic data. Indeed, three mouse models were developed carrying deletions of the 16p11.2 homologous genetic interval [18–20] of slightly different size. Nevertheless, they were found to share common phenotypes including hyperactivity, repetitive behaviours, and deficits in object memory [18–20] that are related to human features.

To find the specific brain regions, developmental periods, networks and pathways impacted by the 16p11.2 deletion, several studies have been carried out. In particular, the development of dynamic spatio-temporal networks of 16p11.2 genes integrating data from brain developmental transcriptome with data from physical interactions of 16p11.2 proteins allowed to support the role of KCTD13 as a protein that complex with CULLIN 3 (Cul3) ubiquitin ligase regulating the Ras homolog family member A (RHOA) protein levels [21]. The known important functions of Rho GTPase signalling pathway in brain morphogenesis at early stages of brain development allowed to propose that *KCTD13* dosage changes in 16p11.2 deletion or duplication carriers influences RHOA levels and lead to impaired brain morphogenesis and cell migration during foetal stages of brain development [21]. This hypothesis was

supported by later studies showing the implication of KCTD13 in the abnormal brain size in downregulation experiments in zebrafish [22].

In addition, $Kctd13^{+/-}$ mouse model showed a reduction of the number of functional synapses, with a decrease of dendritic length, complexity and dendritic spine density due to increased levels of RHOA [23]. These alterations were reversed by RHOA inhibition with rhosin, strengthening the potential role of RHOA as a therapeutic target. Interestingly the recognition deficit was not seen with this model in another more complex paradigm in which the recognition test is based on 3 objects [23]; a more challenging task with multiple step of recognition, involving slightly different mechanisms of memory. Also, recent studies revealed dendritic spine maturation alterations of hippocampal pyramidal neurons in another $Kctd13^{+/-}$ mouse model [24]. This model also presented a deficit in recognition and location memory in a paradigm with two objects similar to the phenotype detected in previous studies done on three distinct 16p11.2 $Del/+$ mouse models [18–20]. Surprisingly, no change in RHOA protein level was detected in the last study [24].

Despite these investigations, how KCTD13 levels can regulate the RHOA signalling pathway, as well as its implication in the phenotypes associated with the 16p11.2 syndromes remain unclear. Thus, we decided to explore the role of $Kctd13$ by engineering a new loss-of-function allele, removing only the exons 3 and 4 with CrispR/Cas9, instead of the whole gene [23] or the exon 2 [24]. Then, we characterized the novel heterozygous mouse model and compared its outcome with the 16p11.2 deletion in a selected number of tests.

Furthermore, the integration of our results with earlier studies led us to hypothesize that the 16p11.2 deletion leads to an over-activation of the KCTD13-CUL3-dependent RHOA pathway. So, if we assume that the RHOA / ROCK pathway over-activation causes behavioural and learning alterations in the 16p11.2 deletion, inhibiting this pathway should improve the $Kctd13^{+/-}$ and the 16p11.2 $Del/+$associated phenotypes. We decided therefore to treat our mice with fasudil (HA1077), an inhibitor of the ROCK kinase from the RHOA pathway. We used a similar strategy to counterbalance the behavioural impairments of the Oligophrenin-1 mouse model of intellectual disability [25]. Thus, we planned first to characterize the behaviour phenotypes of the new $Kctd13$ haplo-insufficient model and to compare the outcome with the 16p11.2 $Del/+$ mice. Then we set up a chronic administration of fasudil (HA1077) in adult $Kctd13^{+/-}$ and 16p11.2 $Del/+$ mice (this work and [20]) and evaluated the behaviour of the treated versus the non-treated animals. This evaluation was carried out, focusing on open field activity and both location and recognition object memory. The novel object location (NOL) memory stimulate the parahippocampal cortex, the entorhinal cortex and the hippocampus [26] whereas the novel object recognition (NOR) memory task is based on the innate preference of rodents to explore novelty involving the perirhinal and entorhinal cortex and the hippocampus. Then we carried a molecular analysis of key members of the RHOA pathway.

Materials And Methods

Mouse lines, genotyping and ethical statement

Two mouse models were used in the study. The 16p11.2 mouse model corresponds to the Del(7*Sult1a1-Spn*)6Yah mouse model[20], noted here *Del/+*. The line was kept on a pure C57BL/6N (B6N) inbred genetic background. The deletion allele was identified by PCR using primers Fwd1 (5'-CCTGTGTGTATTCTCAGCCTCAGGATG-3') and Rev2 (5'-GGACACACAGGAGAGCTATCCAGGTC-3') to detect a specific band of 500 bp while the wild-type allele was identified using Fwd1 and Rev1 (5'-GGACACACAGGAGAGCTATCCAGGTC- 3') primers to detect the presence of a 330 bp fragment. PCR program was: 95 °C / 5 min; 35 × (95 °C / 30 s, 65 °C / 30 s, 70 °C / 1 min), 70 °C / 5 min.

The *Kctd13*^em2(IMPC)Ics knock-out mouse was generated by the CRISPR / Cas9 technology[27] in the B6N genetic background (Sup Fig. 1A). Two pairs of sgRNAs, one pair located upstream and the other pair downstream of the target region, were selected to delete the exon 3 and 4 of the gene. Both pairs of sgRNAs (showing a cut) and Cas9 mRNA were microinjected in fertilized eggs derived from super-ovulated sexually immature B6N female mice (4–5 weeks olds). Injected embryos cultured *in vitro* were implanted into the oviducts of pseudo-pregnant females. The deletion of *Kctd13* in *Kctd13*^em2(IMPC)Ics (noted here *Kctd13*^+/−) was confirmed by PCR using primers Ef (5'-ACCTCTTAGCTGGGCATGCTAAATT-3') and Xr (5'-AGCCTATGCTAACTATTATCACAGG-3') and the sequence of the deleted fragment. PCR reaction gave deletion and wild-type products of 429 and 668 bp long respectively. PCR program was: 94 °C / 5 min, 35 × (94 °C / 30 sec; 60 °C / 30 sec; 72 °C / 30 sec), 72 °C / 5 min. This set of primers was also used for genotyping (Sup Fig. 1B). The model was validated by detecting the decreased level of KCTD13 in the mutant hippocampi compared to control (Sup Fig. 1C). All the mouse models are available through the Infrafrontier European repository or the International Mouse Phenotying consortium.

Chronic fasudil treatment

In this study, we developed a protocol for a pre-clinical treatment (Fig. 1) with the drug fasudil hydrochloride or HA1077 (F4660, LC laboratories Boston, MA, USA). At weaning, control wild type littermates and heterozygous male mice, either *Kctd13*^+/− or *Del/+*, were taken from several litters and housed in groups of 4 – 2 individuals in ventilated cages (Green Line, Techniplast, Italy), where they had free access to water and diet (D04 chow diet, Safe, Augy, France). Animal bedding (Poplar litter, AB 3 autoclavable, AniBed, Pontvallain, France) was changed once a week. At 11 weeks, animals were transferred from the animal facility to the phenotyping area. The temperature was kept at 21 ± 2 °C, and the light cycle was controlled as 12 h light and 12 h dark (lights on at 7 am).

At 12 weeks of age, three independent cohorts of mice for each line, with wild-type (wt) and mutant littermates, were subjected to a battery of behavioural tests (see below) for 2 weeks. Then, 14 weeks old mice were randomly divided into 2 groups: one treated with fasudil administrated orally *ad libitum* in drinking water to reach a dose of 100 mg/kg/day and a second with no treatment. The dose was estimated to 152.7 mg in feeding bottle (250 ml) changed twice a week considering the previous study in which we controlled that the drinking volume was about 4.6 ml/day/mouse [25]. Four weeks after the

beginning of the treatment, 18-week-old mice were challenged once again to the same battery of behavioural tests (see below) and were kept under the same treatment condition (Fig. 1). The experiments were conducted blindly for genotype as recommended by the ARRIVE guidelines [28, 29]. All animals injured by their cage companions were excluded from the behavioural tests at the time when they were seen. A second batch of three independent cohorts were processed similarly but without behaviour test for the molecular analysis of the hippocampal region from treated and non-treated mice. In this case, treated animals started the fasudil treatment at the age of 12 weeks, for 6 weeks. Samples were quickly harvested from 18 weeks-old mice after euthanasia by cervical dislocation and snap frozen for molecular analyses.

Behavioural analysis

We used three tests that previously unravelled robust phenotypes in the three 16p11.2 mouse models [18–20]: the open field for the exploration activity, the novel object location and the novel object recognition for the learning and memory in mice.

For the open field (OF) mice were tested in automated arena (44.3 × 44.3 × 16.8 cm) made of PVC with transparent walls and a black floor, and covered with translucent PVC (Panlab, Barcelona, Spain). The arena was divided into central and peripheral regions (8 cm peripheral zone and 28 cm central zone) and homogeneously illuminated at 150 Lux. Each mouse was placed on the periphery of the open field and allowed to explore the apparatus freely for 30 min. During each session we measured the total distance travelled, evaluated the habituation of the animal over time, by splitting the data in 10-minute intervals and assessed the vertical activity through the number of rears.

The NOL memory task was carried out in the same open field arena as previously described. In the first day, mice were habituated to the arena for 30 min at 150 Lux. On the following day, animals went through an acquisition trial during the first 10 min in which they were individually presented to 2 similar objects A. Each object was placed 10 cm away from each one of the corners on the north side of the box. The exploration time of objects A (when the animal's snout was directed towards the object at a distance ≤ 1 cm) was recorded. Minimum exploration time was set to 3 s, and mice that did not reach this criterion or did not show any interest for one object were excluded from the study. A 10-min retention trial (second trial) was conducted 5 min later, when one of the familiar objects was displaced to a novel location (B) on the south side and the exploration time (t) of the two objects was recorded for 10 min. We used two identical cylindrical objects of black colour with a white circle on top. In this session, minimum exploration time was set also to 3 s, and mice that did not reach this criterion or did not show any interest (0 s of exploration) for one object were excluded from the study. We verified that no preference was seen during the exploration of the left and right object. The recognition index (RI) was defined as (tB / (tA + tB) × 100). A RI of 50% corresponds to chance level and a significantly higher RI reflects a good recognition of which object was moved in between the two sessions.

The NOR test was performed in a circular open field of PVC white with opaque walls and floor of 30 cm high and 50 cm diameter. On the first and second days, each mouse was habituated to the arena for 15

minutes at 60 Lux. The following day, we started the NOR sessions. First, each animal was individually given a 10 minutes acquisition trial for the presentation of two identical objects A (either marble or dice) placed at the northeast or northwest of the open field arena. The exploration time of both objects A was recorded. 3 hours later (retention delay in home cages), a 10 minutes retention trial (second trial) was performed. One of the identical object A was replaced with a novel object B at the same position. The exploration time of the two objects (familiar object and novel object) was recorded. The recognition index (RI) was defined as (tB / (tA + tB) × 100). A RI of 50% corresponds to chance level and a significantly higher RI reflects good recognition memory. All mice that did not explore the objects for more than 3 seconds during the acquisition trial or the retention trial or did not show any interest for one object were excluded from the analysis.

Western blot

Fresh hippocampal tissues were isolated by rapid decapitation/dissection of naive mice and snap frozen. Then, they were lysed in ice-cold sonication buffer supplemented with Complete™ Protease Inhibitor Cocktail (Roche). Individual samples were disaggregated, centrifuged at 4 °C for 30 minutes at 14000 rpm, diluted in 4X Laemmli sample buffer containing β-mercaptoethanol (Bio-Rad, France), and incubated at 95 °C for 5 min. Protein concentration was determined by Pierce™ BCA Protein Assay Kit (23225, Thermo Fisher Scientific, Strasbourg). Samples were diluted with sample buffer such that 30 µg of protein were loaded per lane onto 15% polyacrylamide gel. Gels were run and then transferred to nitrocellulose membranes by Trans-Blot® Turbo™ Transfer System (BioRad, France) through MIXED MW Bio-Rad Preprogrammed Protocol. Then they were blocked in 5% BSA, 1 X Tris-buffered saline, 0.1% Tween 20 (TBS-T) and incubated with primary antibody during 10 minutes. Membranes were washed in TBS-T followed by a 10 minutes secondary antibody incubation using an HRP conjugated Goat anti-Rabbit IgG (A16096, Invitrogen, France) at 1:5,000 through SNAP i.d.® 2.0 Protein Detection System (C73105, Merck). This apparatus has a vacuum-driven technology and a built-in flow distributor that actively drives reagents through the membrane.

Total levels of KCTD13, RHOA protein and Myosin Light Chain and its phosphorylation product were analysed using Western Blot. Proteins were visualized with Amersham™ Imager 600. Signals were quantified using ImageJ and analysed using Microsoft Excel and GraphPad Prism. We used the following primary antibodies: KCDT13 (dilution 1:250, HPA043524, Atlas Antibodies, Bromma, Sweden), RHOA (dilution 1:1,000; cat #2117, Cell Signaling, USA), MLC (dilution 1:1,000;cat # 8505, Cell Signaling Technology Europe, B.V., Leiden, The Netherlands) and pMLC (dilution 1:1,000; Thr18/Ser19 cat #3674, Cell signalling, Boston, MA, USA). The ratio of protein (or phosphorylated protein) level was estimated against control β-actin protein level (detected with a mouse monoclonal Anti-β-Actin – Peroxidase antibody (A3854 Sigma)) and normalized to untreated wt sample mean.

Statistical analysis

The statistical analysis was carried out using standard statistical procedures available on the SigmaPlot software (Systat software, San Jose, USA). All outliers were identified using the Grubbs' test from

calculator GraphPad (GraphPad Software, San Diego) or ROUT method with a Q value of 1% from GraphPad Prism 7.01 (GraphPad Software, San Diego) when data with nonlinear regression. We estimated a priory the power of the different variables, scored during the behavioural analysis, to test our H0 hypothesis taking into account our previous analysis of the Del/+ model [20]. A posteriori we verified that the sample size was sufficient to have a statistical power of 100% for the object location and recognition memory at both age (Sup. Table 1). Nevertheless the power of the open field variable "distance travelled" were lower at 12 compared to 18 weeks, certainly due to change in variability. Based on the power calculation we did not consider the results of the rearing activity from the open field. Data from behavioural characterization of $Kctd13^{+/-}$ and 16p11.2 $Del/+$ mouse models were analysed through the Student t-test (see supplementary table 2 and table 3 as a summary). One sample t-test was used to compare recognition index values to the set chance level (50%). Data from post-treatment behavioural phenotyping of both genetic lines were analysed using one-way ANOVA followed by Tukey's post-hoc test whenever data presented normal distribution and equal variance. Otherwise, we used the non-parametric Kruskal-Wallis one-way analysis of variance and Mann-Whitney U test. One sample t-test was used also to compare recognition index values to the set chance level (50%). Western blot data were analysed using Kruskal-Wallis one-way analysis of variance test between groups followed by Mann-Whitney U test or Student t-test depending on data distribution. Data are represented as the mean ± SEM and the significant threshold was $p < 0.05$.

Results

Phenotypic characterization of the new Kctd13$^{+/-}$ mouse model and comparison with the 16p11.2 Del/+ mouse model

We created a new $Kctd13$ KO mouse model with the deletion of exon 3 and 4, leading to a frameshift at the transition of exon 2 and 5 (Sup Fig. 1A). We have validated the new allele by DNA analysis for the deletion, the decrease expression of KCDT13 in mutant hippocampi (Sup Fig. 1) and by phenotyping at the IMPC (www.mousephenotype.org). Then, we evaluated its learning and memory behaviour at 12 weeks of age in the open field, NOL and NOR, and compared the results obtained with the characterization of 16p11.2 $Del/+$ model (Fig. 2). First, we did not detect significant differences in the open field test (12 weeks), when we measured the total distance travelled by $Kctd13^{+/-}$ mice compared to wt littermates. This result was different from the phenotype of the $Del/+$ mice (Fig. 2A). Indeed $Del/+$ mutant mice were more active with a significant increase in the distance travelled compared to their wt littermates as previously observed [20]. Then, we analysed the distance travelled in 5-minute intervals, to see the habituation to a new environment during the test. We found that the $Kctd13^{+/-}$ mice experience a similar habituation to the control individuals (Sup Fig. 2A). Here too, a significant difference was seen in the 16p11.2 $Del/+$ carrier mice. Athough being more active, the mutant mice showed normal habituation compared to their wild-type littermates.

Afterwards, we analysed the object location memory (Fig. 2B). For this test, animals were scored for their ability to distinguish between a previously presented object that stays in the same location and another

object whose position was changed, after a retention delay of 5 min. The recognition indexes for the novel location object showed that, contrary to their wt littermates, both $Kctd13^{+/-}$ and 16p11.2 $Del/+$ mice were not able to differentiate between the novel and familiar location.

Finally, we investigated whether $Kctd13^{+/-}$ mice could discriminate a novel object from a previously explored set of two objects after a retention delay of 3 hours in the NOR task (Fig. 2C). Whereas wt animals were able to differentiate objects showing a novel object preference, $Kctd13^{+/-}$ mice were not able to discriminate the novel from the familiar object. The deficit was like the one seen in the 16p11.2 Del/+ mice. Overall, our behavioural analysis showed that the $Kctd13$ haploinsufficiency, in the pure C57BL/6N genetic background, phenocopied the object location and recognition memory deficits seen in the 16p11.2 deletion model. However, the increased exploration activity found in the 16p11.2 $Del/+$ mice was not seen in the $Kctd13^{+/-}$ mutant mice.

Fasudil treatment partially reverses the cognitive impairment in the Kctd13$^{+/-}$ and in the 16p11.2 Del/+ mouse models

Thus, after the behavioural characterization of the $Kctd13^{+/-}$ and 16p11.2 $Del/+$ mouse models, at 14 weeks of age, individuals from both genotypes were randomly assigned to a subgroup that was treated with fasudil or an untreated control group for 4 weeks prior to further behaviour testing (Fig. 1). First, we noticed that the fasudil treatment did not change the locomotor exploration activity of the different genotypes (Fig. 3A; Sup Fig. 3A) with Del/+ individuals travelling more than the control wt with no effect of the treatment (Fig. 3B, Sup Fig. 3B). Similar to earlier stage, the 18weeks old $Kctd13$ mutant mice were no more active in the open field than their control littermates. Interestingly, one month after the first phenotypic characterization, $Kctd13^{+/-}$ mice performed in the NOL (Fig. 3B) but with a lower recognition index compared to wt. In contrast, no improvement was observed at 18 weeks of age in 16p11.2 $Del/+$ mice (Fig. 3B). However, fasudil treatment significantly improved the NOR defect found in both $Kctd13^{+/-}$ and 16p11.2 $Del/+$ mice at 19 weeks of age (Fig. 3C). Here the altered recognition memory, found previously at 12 weeks, was seen again in mutants at 19 weeks,and the fasudil treatment had a rescuing effect both in the $Kctd13^{+/-}$ and the 16p11.2 $Del/+$ models. It also highlights that, at least one gene, deleted in the 16p11.2 $Del/+$ model, and different from $Kctd13$, contributes to the NOL phenotype in this model at this latter age.

Molecular analyses of RHOA / ROCK signalling pathway in the Kctd13$^{+/-}$ and the 16p11.2 Del/+ mouse models

Then we checked whether the RHOA / ROCK signalling pathway was changed in both 16p11.2 deletion and $Kctd13$ mouse models. Neither the loss of one copy of the complete chromosomic region in 16p11.2 $Del/+$ mice nor the deficiency of $Kctd13$ resulted in increased levels of RHOA in the hippocampal region at 18 weeks of age (Fig. 4A-B), nor at a change of MLC level in $Kctd13$ mutant versus wt control (Sup. Figure 4) Nevertheless, both models showed an over activation of the RHOA / ROCK pathway. Indeed, the level Myosin Light Chain (MLC), a protein targeted by the RHOA/ROCK pathway, was not showed

increased levels of phosphorylation in the hippocampus of these mice, supporting the idea that this pathway could be associated to the cognitive phenotype observed (Fig. 4C and D; Sup table 4). Therefore, we verified whether fasudil therapeutic effect was acting through the inhibition of this RHOA/ROCK signalling pathway. Interestingly, the fasudil treatment reduced MLC phosphorylation levels in *Kctd13* mutant individuals. As for the *Kctd13*$^{+/-}$ model, we found that in 16p11.2 deficient mice fasudil restored a normal MLC phosphorylation in treated mutant mice but surprisingly induced increased MLC phosphorylation in wt mice.

Discussion

Here, we described the phenotypes of another *Kctd13*$^{+/-}$ mouse mutant line that replicates some of the defects seen in young mouse models carrying the 16p11.2 BP4-BP5 deletion and we explored a treatment aiming at reducing the activity of the RHOA / ROCK pathway on cognition. With the new *Kctd13*$^{+/-}$ mouse line, we were able to detect changes in the NOL and NOR but no effect on the exploration activity compared to the 16p11.2 *Del/+* mouse model. These results are in agreement with a recent study highlighting the role of KCTD13 in the 16p11.2 deletion syndromes [24]. The loss of one copy of *Kctd13* gene did not cause alterations on exploration or vertical activity of mice in open field test. However, the hemi-deletion of entire 16p11.2 region induced hyperactivity in these animals. This observation leads us to propose that *Kctd13* genetic dosage is not involved in the increased exploration activity associated with 16p11.2 deletion. Accordingly, chronic fasudil administration did not attenuate the hyper locomotion affecting 16p11.2 *Del/+* mice. This finding suggests that there could be other genes of the region involved in this phenotype. For this reason, the treatment with an inhibitor of the RHOA / ROCK pathway did not produce any effect on this phenotype.

When we analysed the NOL in *Kctd13* mutant mice, we found an impairment in the novelty detection in NOL test at 12 weeks of age. However, at 18 weeks when we repeated the test, the non-treated mutant mice showed an improved recognition index. This observation may show that either there is a maturation deficit that is recovered in older mice, may be through compensatory effects, or there could be an effect in the repetition of the test. We favour the first hypothesis as the NOR/NOL phenotypes in this line were found at both ages in the 16p11.2 deletion. Indeed, we found a NOL deficit for the 16p11.2 *Del/+* mice at 12 weeks, that was still present at 18 weeks, not corrected by chronic administration of fasudil for 5 weeks. So, future research will be necessary to analyse how this *Kctd13*-induced NOL phenotype is rescued in 18-week-old no-treated mice. This finding confirms that the NOL-phenotype associated to 16p11.2 rearrangement is not completely dependent on *Kctd13* dosage. Several genes can contribute to the NOL phenotype on top of the *Kctd13* gene. Indeed, loss of the serine/threonine Tao kinase 2 leads to brain and behavioural abnormalities with altered dendritic growth and synaptic connectivity through RHOA signalling [30].

The object recognition memory deficit is one of the most robust and reproducible phenotypes associated with 16p11.2 *Del/+* mice [20]. In agreement with precedent research, our *Kctd13*$^{+/-}$ mouse model has deficits in novelty detection. Furthermore, our study showed that fasudil treatment significantly rescued

this impairment in mutant mice. Thus, loss of KCTD13 is a main driver of object recognition phenotype associated with 16p11.2 deletion. In addition, the therapeutic effects of fasudil on *Kctd13* deficient and 16p11.2 deletion mice highlight the role of the RHOA/ROCK signalling pathway as the main mechanism responsible of this phenotype.

Interestingly deficient individuals for the *Kctd13* gene showed no change in expression levels of the RHOA protein and the fasudil treatment did not modify RHOA protein expression in mutant and control mice. Likewise, the carriers of the 16p11.2 hemi-deletion did not display changes of expression for this protein. Even if we were not able to detect a change in the RHOA protein level, *Kctd13* mutants as well as 16p11.2 deficient mice had increased phosphorylated-MLC levels. This observation confirms that RHOA / ROCK pathway outcome is over-activated due to a loss copy of *Kctd13* in *Kctd13* and 16p11.2 mutants. Furthermore, our study showed that the therapeutic effect of fasudil in recognition memory phenotype associated to 16p11.2 deletion was somehow due to the normalizing action of the drug in both mouse models.

At this point, we can highlight the clinical relevance of treatment because of its potential as a cognitive enhancer in humans with memory and learning dysfunction related to neurodevelopmental disorders. However, more work is necessary to understand the precise molecular mechanism affected by the loss of the *Kctd13* gene that is causing the over-activation of the RHOA / ROCK pathway.

We consider that further biochemical studies of the RHOA / ROCK pathway and the KCTD13-CULLIN3 complex are needed to understand the mechanistic role of the pathway in the syndromes associated with 16p11.2 rearrangements. In addition, our results do not prevent that other genes in the region may act at other levels of the RHOA pathway or at different times in development [30]. Probably several genes play a similar role, or the expression of a gene may regulate the expression of another gene in the region, considering the high genotypic density of the 16p11.2 interval and the variability of neurological-associated phenotypes [31, 32].

Limitations

In this study we found some similarities between *Kctd13* and 16p11.2 deletion models not only in terms of phenotypes but also in terms of rescue for the novel object location and object recognition paradigms. This analysis is limited as it has been done in a pure genetic background where some of the Del/+ mutants are not viable. Thus, this study has been limited to a subset of viable mutant animals carrying the 16p11.2 deletion. We could have improved this because those models can display a normal post-natal viability on a F1 genetic background with also defect in social behaviour [20]. Nevertheless, we would have to do a similar study with the *Kctd13* null allele. Overall, this study was made on mouse model that only replicate a partial set of features found in people with 16p11.2 deletion thus our conclusion only bring a partial response for the human condition.

Conclusion

Here we showed that *Kctd13* haploinsufficiency phenocopied the object location and recognition memory deficits seen in the 16p11.2 deletion model but did not change the exploration activity in the open field. Treatment in adult mice with fasudil, an inhibitor of RHOA, was able to restore novel object location and recognition memories. Furthermore, fasudil treatment was able to restore RHOA/ROCK signalling pathway to almost normal level of phosphorylation of the Myosin Light Chain in the brain. Altogether targeting the RHOA pathways may be an alternative for improving cognition in people carrying the 16p11.2 deletion.

Abbreviations

Del
deletion
MLC
myosin light chain
NOL
novel object location
NOR
novel object recognition
OF
open field
RI
recognition index
wt
wild-type

Declarations
Consent for publication

Not applicable

Ethics approval

Experimental procedures involving animals were approved by the Ministry of National Education, Superior Learning and Research and with the agreement of the local ethical committee Com'Eth (n°17) under the accreditation number APAFIS#3590-2016011510199843 v4 with YH as the principal investigator (accreditation 67-369).

Availability of data and materials

All the mouse models are available either through the Infrafrontier research infrastructure (16p11.2 deletion, www.infrafrontier.eu; EM:06133) or the international mouse phenotyping consortium (Kctd13 mutant, www.mousephenotype.org).

The datasets analysed during the current study are available from the corresponding author on reasonable request.

Competing interest

The authors declare that they have no conflict of interest.

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author's contributions

SML, MCB, YH: contributions to the conception or design of the work;

SML, YH: Drafting the work or revising it critically for important intellectual content

SML, VN, CC, YH: contribution to the acquisition, analysis, or interpretation of data

SML, VN, CC, MCB, YH: Final approval of the version to be published;

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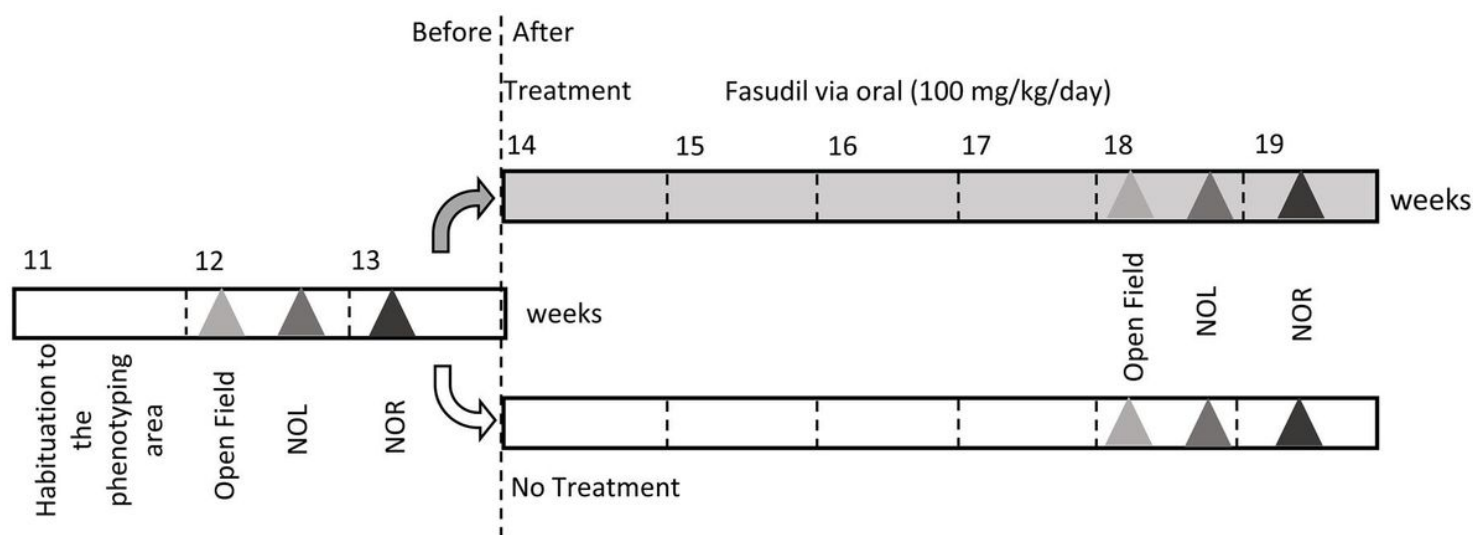
Figures

Figure 1

Outline of the behavioral pipeline used for investigating the therapeutic effects of fasudil drug in Kctd13+/- and 16p11.2 Del/+ mouse models. We used 3 independent subcohorts of animals for each mouse line. 12-week-old mice were subjected to different behavior and learning tests, after a previous habituation to the phenotyping zone. At 14 weeks, each cohort was divided into two groups. The first

group started the fasudil treatment and the second group followed the cognitive characterization without treatment. NOL: novel object location, NOR: novel object recognition.
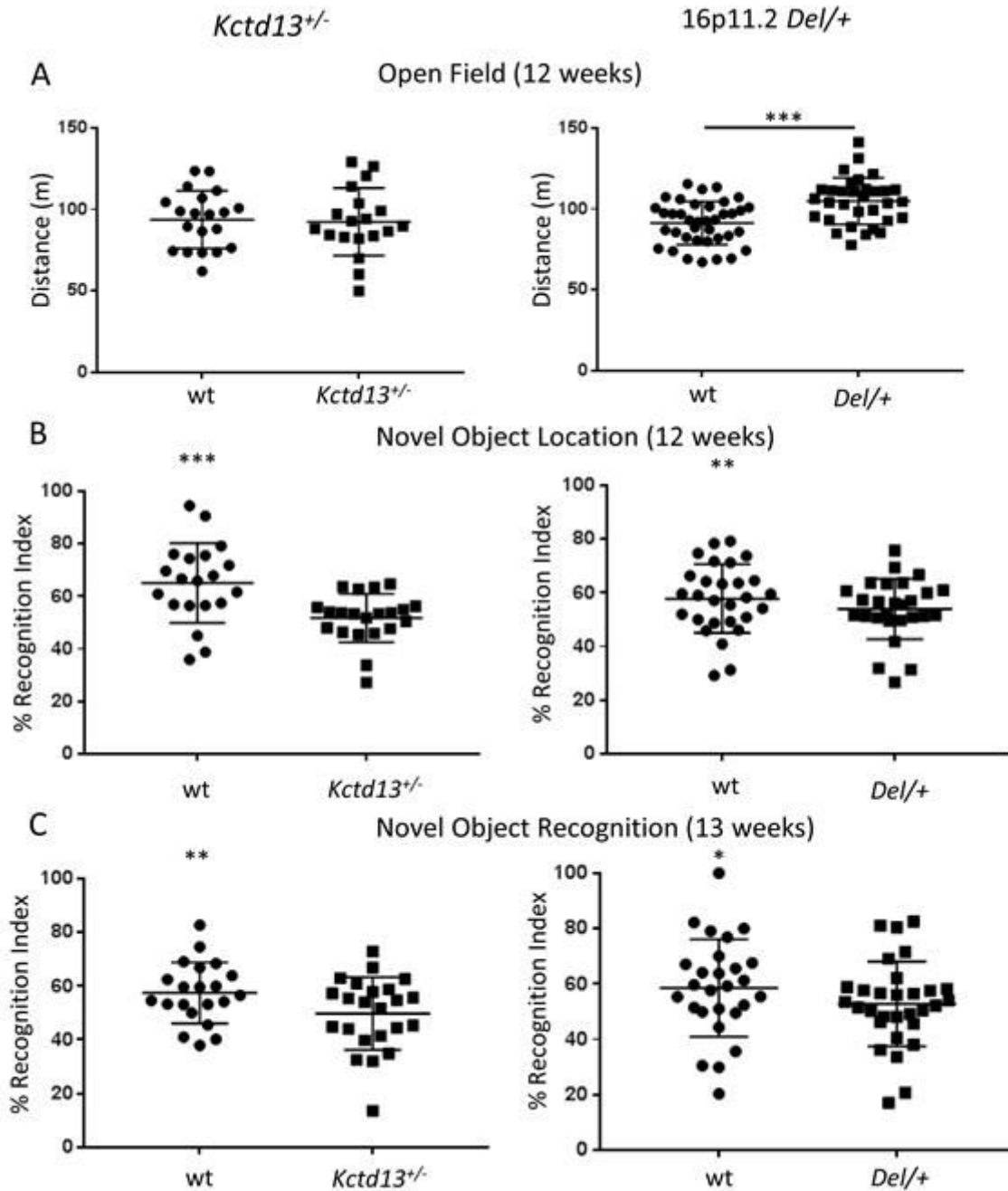

Figure 2

Exploration activity in the (A) Open field test, (B) Novel object location and (C) novel object recognition of the Kctd13+/- and the 16p11.2 Del/+ mouse models at 12 and 13 weeks of age. (A) Male mice from the Kctd13+/- line (wt (n=20) and Kctd13+/- (n=19)) or from the 16p11.2 Del/+ line (wt (n=38) and Del/+ (n=32)) were free to explore the open field for 30 min as a new environment. First, the exploration activity was analyzed from the total distance traveled during the test. Next, the adaptation of the mice to the environment was evaluated by dividing the test into periods of 10 minutes. The Kctd13+/- animals showed no alteration compared to their wt littermate whereas the 16p11.2 Del/+ mice showed increase

exploratory activity in the distance travelled compared to wt (Student t test: Total distance: wt vs. Del/+ t(68) = -4.096; p < 0.001). The Del/+ mutant individuals displayed a decreased of the arena exploration during the test and thus a normal habituation to the new environment (Paired t-test: T0-10 vs. T20-30 t(31) = 14,319; p < 0.001). (B) In the NOL test, the recognition index reflects the ability of mice from the two lines (wt (n=20) and Kctd13+/- (n=21) littermates; and 16p11.2 Del/+ (wt (n=28) and Del/+ (n=27) littermates) to distinguish the new located object from the familiar one after a 5 min retention delay. The Kctd13+/- and the Del/+ male mice showed a deficit in object location recognition memory compared to their wt littermates (One sample t-test for the Kctd13+/- line: wt (t(19) = 4.4607; p = 0.0003), Kctd13+/- (t(16) = 0.9170; p = 0.3728); and the 16p11.2 Del/+ model: wt (t(27) = 3.2299; p = 0.0032), Del/+ (t(26) = 1.8372; p = 0.0776)). (C) In the NOR test, the animals (wt (n=21) and Kctd13+/- (n=23) littermates; and wt (n=27) and Del/+ (n=30) littermates) were challenged to recognize the new object from the familiar object after a 3 h delay. Kctd13+/- and the Del/+ mice showed a poor object recognition memory compared to their respective wt littermates (One sample t-test for the Kctd13+/- model: wt (t(20) = 3.0179; p = 0.0068), Kctd13+/- (t(22) = 0.0805; p = 0.9366); and the 16p11.2 Del/+ model: wt (t(26) = 2.5312; p = 0.0178), Del/+ (t(29) = 1.0119; p = 0.3199)). (* p < 0.05; ** p < 0.01; *** p < 0.001).
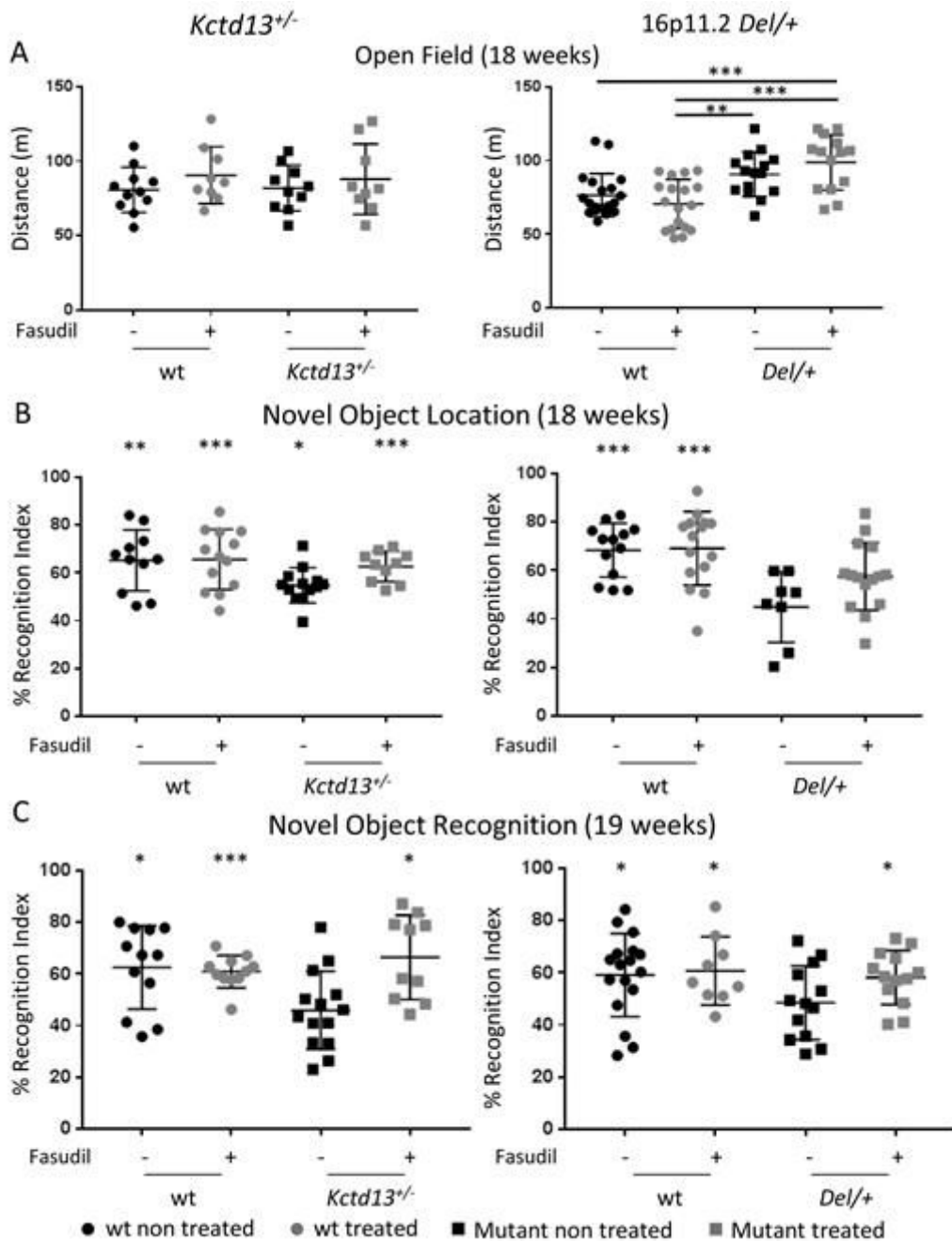
Figure 3

Exploration activity in the (A) Open field test, (B) Novel object location and (C) novel object recognition of the Kctd13+/- and the 16p11.2 Del/+ (B) mouse models with or without fasudil treatment. (A) For the, male mice from the Kctd13+/- line (non-treated wt (n=11), treated wt (n=9), non-treated Kctd13+/- (n=10) and treated Kctd13+/- (n=9)) or from the 16p11.2 Del/+ line (non-treated wt (n=20), treated wt (n=18), non-treated Del/+ (n=15) and treated Del/+ (n=14)) explored the open field for 30 min as a new environment. The exploration activity was measured with the total distance traveled during the test. Next, the habituation of the mice to the environment was evaluated by dividing the test into periods of 10 minutes. The Kctd13+/- animals showed no alteration compared to their wt littermate whereas the Del/+ mice showed increase exploratory activity in the distance travelled, which was not affected by the fasudil

treatment (One way ANOVA between groups: F(3,63) = 10.158; p < 0.001; Tukey's post hoc tests: non treated wt vs. treated wt : p = 0,675, non-treated Del/+ vs. treated Del/+ : p = 0.54, non-treated wt vs. treated Del/+ : p = 0.001, treated wt vs. non treated Del/+ : p = 0.004 and treated wt vs. treated Del/+ : p < 0.001). Nevertheless, treated Del/+ individuals experienced a decreased activity less pronounced throughout the test (Paired t-test: T0-10 vs. T20-30 t(13) = 2.010; p = 0.066). The treatment did not have either effect for any genotype on the vertical activity (Kruskal-Wallis one-way analysis of variance : H(3) = 7.074; p = 0.070). (B) For NOL test, recognition index reflects the ability of mice from the two lines Kctd13+/- (non-treated wt (n=11), treated wt (n=13), non-treated Kctd13+/- (n=13) and treated Kctd13+/- (n=10) littermates) and the 16p11.2 Del/+ (non-treated wt (n=13), treated wt (n=15), non-treated Del/+ (n=8) and treated Del/+ (n=15) littermates) to distinguish the new location of an object from the familiar one after a 5 min retention delay. We observed again that the 16p11.2 Del/+ male mice showed a deficit in object location memory compared to their wt littermate but the Kctd13 heterozygotes were no longer defective (One sample t-test: non treated wt (t(10) = 3.9679; p = 0.0027), treated wt (t(11) = 5.3506; p = 0.0002), non-treated Kctd13+/- (t(12) = 2.3628; p = 0.0359), treated Kctd13+/- (t(8) = 5.7266; p = 0.0004)) and the fasudil treatment was not able to restore this ability in the 16p11.2 Del/+ model (One sample t-test: non-treated wt (t(11) = 5.4167; p = 0.0002), treated wt (t(13) = 4.4492; p = 0.0007), non-treated Del/+ (t(7) = 0.9837; p = 0.3580), treated Del/+ (t(14) = 2.1021; p = 0.0541)). (C) In the NOR test, the mutant animals from the Kctd13+/- (non-treated wt (n=12), treated wt (n=11), non-treated Kctd13+/- (n=14) and treated Kctd13+/- (n=10)) or the 16p11.2 Del/+ model (non-treated wt (n=17), treated wt (n=9), non-treated Del/+ (n=13) and treated Del/+ (n=13)) were challenged to recognize the new object from the familiar object after a 3 h delay. Kctd13+/- and the Del/+ mutant mice were both impaired to recognize the new object compared to their respective wt littermates in the non-treated group and the fasudil treatment was able to restore the object recognition in the Kctd13+/- line (One sample t-test: non-treated wt (t(11) = 2.6929; p = 0.0209), treated wt (t(10) = 5.7297; p = 0.0002), non-treated Kctd13+/- (t(11) = 2.6101; p = 0.0243 (less than 50%), treated Kctd13+/- (t(9) = 3.1937; p = 0.0109)) and in the 16p11.2 Del/+ model ((One sample t-test: non-treated wt (t(16) = 2.3736; p = 0.0305), treated wt (t(8) = 2.4481; p = 0.0401), non-treated Del/+ (t(12) = 0.3787; p = 0.7115), treated Del/+ (t(10) = 2.9168; p = 0.0154)). (* p < 0.05; ** p < 0.01; *** p < 0.001).
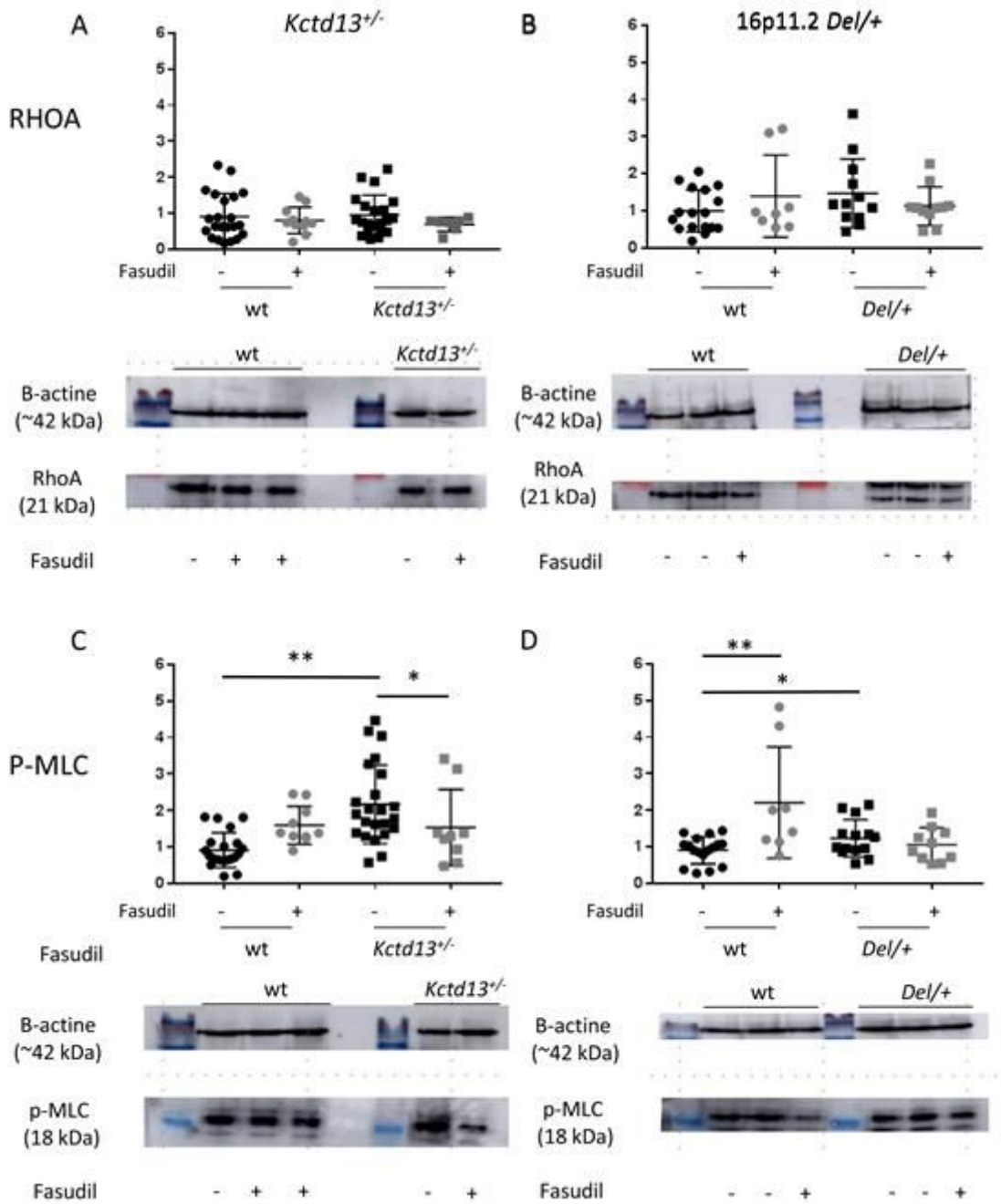
Figure 4

Detection of RHOA (A, B) and of the phoshorylated form of MLC (P-MLC, C,D) by western blots in heterozygous Kctd13+/- (A,C) and the 16p11.2 Del/+ (B,D) hippocampal lysates and their control (wt) littermate. (A) The quantification of the western blot (an example is shown below the graph) revealed no changes in RHOA protein levels in the Kctd13+/- (A) or in the 16p11.2 Del/+ (B) mutant lines compared to their wt littermates. Fasudil treatment did not cause changes in RHOA protein levels in the two mutant lines (non-treated wt (n=22), treated wt (n=11), non-treated Kctd13+/- (n=21) and treated Kctd13+/- (n=7); and non-treated wt (n=17), treated wt (n=8), non-treated Del/+ (n=11) and treated Del/+ (n=11)). However, Kctd13 deficient mice showed an increase in the levels of phosphorylated MLC protein (C) and the loss of a copy of 16p11.2 region caused an increase in the levels of phosphorylated MLC protein (D). The

treatment with fasudil reversed this alteration in Kctd13+/- (non-treated wt (n=21), treated wt (n=10), non-treated Kctd13+/- (n=23) and treated Kctd13+/- (n=9)) (Kruskal-Wallis one-way analysis of variance between groups : H(3) = 21.731; p < 0,001; Mann-Whitney Test : non treated wt vs. non treated Kctd13+/- : p = 0.009; non treated wt vs. treated Kctd13+/- : p = 0.702; non treated Kctd13+/- vs. treated Kctd13+/- : p = 0.049) and in the Del/+ mutant line (non-treated wt (n=17), treated wt(n=8), non-treated Del/+ (n=14) and treated Del/+ (n=10)) (Kruskal-Wallis one-way analysis of variance between groups : H(3) = 8.457; p = 0.037; Mann-Whitney Test : non treated wt vs. treated wt : p = 0.008; t-test : non treated wt vs. non treated Del/+ : p = 0.047, non -reated wt vs. treated Del/+ : p = 0.364). (* p < 0.05; ** p < 0.01).

Supplementary Files

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