Figure 15. Characterization of IL-6 pathway in symptomatic HET mice. **(A-C-F-H)** Graphs show the level of expression of *IL-6* and *Stat3* mRNAs in P100 **(A, F)** and P200/250 **(C, H)** cortex (CTX) and hippocampus (HP) of WT (set at 100%) and HET animals. **(E)** The graph represents the correlation between *Stat3* and *IL-6* mRNA levels in P200/250 HET mice cortices, evaluated by Pearson's correlation test ($p = 0.062$). Pearson r value indicates a direct correlation between the expression of the two genes ($r = 0.47$). **(B-D-G-I)** Histograms show the pStat3 and Stat3 protein levels normalized on the total protein content of the samples. Representative bands of pStat3, Stat3 and the total protein content visualized by TGX stain-free technology are shown on the right. All the data in above mentioned graphs are expressed as mean ± SEM and statistics is performed with Student's t-test or Mann-Whitney test according to data distribution (*$p<0.05$).

4.4 Alterations in cholesterol pathway in *Mecp2* KO astrocytes affect synaptic phenotype

4.4.1 Cholesterol biosynthesis and transport are impaired in *Mecp2* KO astrocytes

As described in preliminary data section, treatment of neurons with KO ACM is sufficient to cause a significant decrease in the number of active synapses, indicating that other factors strictly related to the astrocyte genotype participate to the onset of synaptic defects. Indeed, we believe that the observed defect could be caused both by the release of synaptotoxic factors and/or the lack of beneficial cues. To unveil the contribution of *Mecp2* deficiency in the alterations of ACM composition, we started focusing on one of the first described synaptogenic molecules, mainly released by astrocytes in adult brain, that is cholesterol (132,172). Interestingly, many data point to a defective metabolism of this lipid in RTT models (100,173–176). Therefore, we decided to explore whether defects in cholesterol metabolism are present in primary cultures of *Mecp2* null astrocytes. By qRT-PCR, we found a general downregulation of genes coding for proteins involved in cholesterol biosynthesis, namely *Nsdhl*, *Hmgcr*, *Mvk* and *Sqle*, in KO astrocytes. Additionally, we observed a significant decrease in the expression of *Lcat*, *Abcg1* and *Abca1*, which are related to cholesterol transport. Conversely, the expression of *ApoE*, a gene coding for a protein responsible for cholesterol trafficking to neurons, shows no difference between the two experimental groups (Figure 16A). To gain insight on the putative involvement of cholesterol

metabolism in a more comprehensive model, we evaluated the expression of the same genes in astrocytes isolated by MACS sorting from P7 mice cortices, highlighting a decrease in the level of *Hmgcr* and *Nsdhl* in *Mecp2* KO cells (Figure 16B). Notably, some genes that are significantly downregulated in KO astrocyte mono-cultures, such as *Mvk*, *Sqle, Lcat*, *Abcg1* and *Abca1,* do not show differential expression in MACS-sorted astrocytes, confirming that gene expression in astrocytes is influenced by the interactions with other cell types. Continuing our investigation, we focused on the *Nsdhl* gene, which we found consistently and significantly decreased in *Mecp2* KO astrocytes. Nsdhl encodes for a crucial sterol dehydrogenase involved in cholesterol biosynthesis and is known to be downregulated both in RTT patients and animal models (177,178). By western blot, we observed that the expression of Nsdhl protein is almost halved in KO astrocytes with respect to WT (Figure 16C), coherently with the significant decrease of *Nsdhl* mRNA levels recorded in KO mono-cultures (Figure 16A).

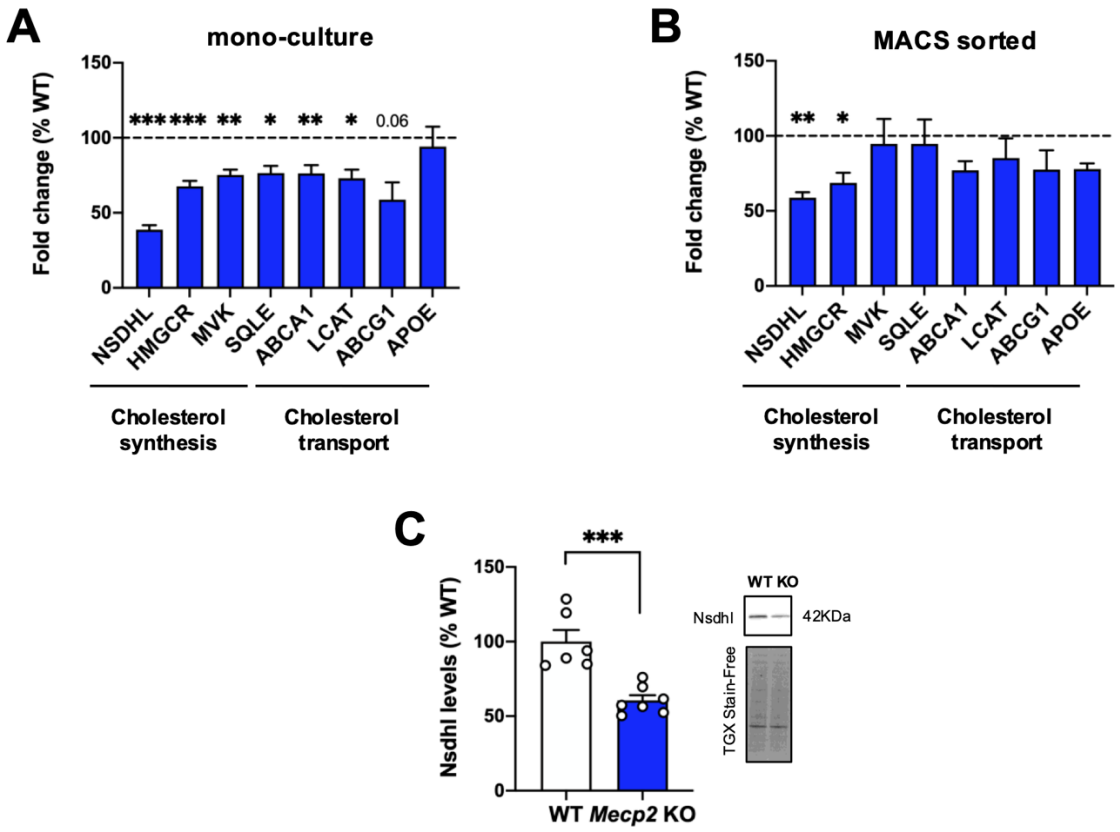

Figure 16. Expression of factors involved in cholesterol pathway is altered in Mecp2 KO astrocytes. **(A-B)** Histograms show the expression of genes involved in cholesterol biosynthesis and transport in primary cultures of *Mecp2* KO astrocytes (n≥5) **(A)** or in KO astrocytes sorted with MACS technology (n≥5) **(B)** with respect to WT astrocytes (set at 100% and represented by dotted line). **(C)** Graph represents the protein level of Nsdhl in western blot

of WT (set at 100%) and KO astrocytes, normalized on the total protein content of the samples. Representative bands of Nsdhl and the total protein content visualized by TGX Stain-Free technology are shown on the right. All the data in above mentioned graphs are expressed as mean ±SEM and statistics is performed with Student's t-test or Mann-Whitney test according to data distribution (*p<0.05, **p<0.01, ***p<0.001). Analyzed samples come from at least 2 independent experiments.

4.4.2 Trofinetide treatment of *Mecp2* KO astrocytes ameliorates defective expression of cholesterol-related genes

To date, the only approved treatment for RTT is Trofinetide, an analog of the N-terminal tripeptide of IGF-1 (GPE) (55,56,179,180). Considering previous evidence reporting the effectiveness of GPE and IGF-1 on neurons when co-cultured with *Mecp2* mutant astrocytes (151), we investigated whether Trofinetide could affect the expression of genes associated with cholesterol metabolism in astrocytes. WT and KO astrocytes were treated for 72 hours with of Trofinetide (50 ng/mL) or empty medium as control. By qRT-PCR, we found that the significant reduction of the expression of *Abca1*, *Mvk* and *Abcg1* genes reported in KO astrocytes is no more present after treatment (Figure 17C-17E). Conversely, the strong decrease of Nsdhl expression is not rescued by Trofinetide (Figure 17A). The data demonstrate that a drug already proven to have beneficial effects on RTT patients also modulates the expression of cholesterol-related genes, further highlighting other molecular targets and the potential role of cerebral cholesterol in the disease.

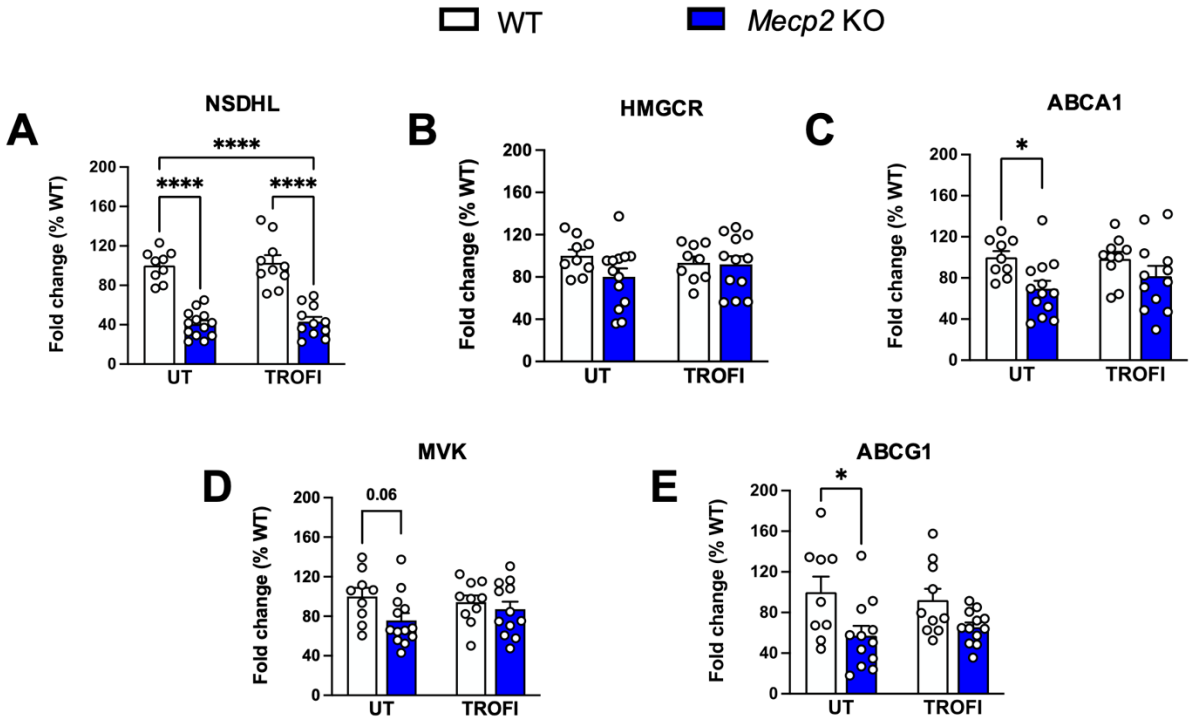

Figure 17. The expression of some of the deregulated cholesterol-related genes in KO astrocytes is recovered after Trofinetide treatment. Histograms show the expression of genes involved in cholesterol biosynthesis and transport in mono-cultures of WT and *Mecp2* KO astrocytes, treated with Trofinetide (TROFI) or vehicle (UT) for 72 hours. Mean expression of the genes in WT UT astrocytes is set at 100%. Data are expressed as mean ±SEM and statistical analysis is performed by two-way ANOVA followed by Sidak's post hoc test (*p<0.05, ****p<0.0001). Data are collected from at least 3 independent experiments.

4.4.3 Cholesterol supplementation rescues synaptic defects

Having observed a general downregulation of genes related to cholesterol pathway in KO astrocytes, indicative of an alteration in its metabolism, we proceeded to assess whether this defect could contribute to the synaptic impairment induced by KO ACM. We supplemented the ACM with exogenous cholesterol during the 24 hours treatment on neurons. We exploited a water-soluble cholesterol formulation complexed to methyl-b-cyclodextrin that can be easily internalized by cells (172,181,182). The first step was to establish a safe dose of cholesterol that could be well tolerated from neuronal cultures. Neurons were treated with increasing doses of the drug from DIV13 to DIV14 and cell viability was evaluated by MTT assay. The analysis revealed a dose-dependent effect, with cholesterol concentrations at or below 5 µg/mL resulting in less than 20% of cell death (Figure 18A). Thus, we treated neurons with WT and KO ACM supplemented with water soluble cholesterol to a final concentration of 0.1 µg/mL. WT

and KO ACM with no cholesterol addition are used as controls. Immunofluorescence staining of pre-synaptic (Synapsin1/2) and post-synaptic (Shank2) markers confirmed the already reported defect induced by KO ACM on neurons, and also unveiled that the addition of cholesterol is sufficient to rescue synaptic alterations (Figure 18B-18E). Encouraging results were also obtained in HET neurons, that are known to show severe synaptic alterations, as KO neurons (183). Indeed, we found that defects in the density of pre- and post-synaptic proteins puncta and in their colocalization are recovered by cholesterol directly supplemented in the culture medium (0.1 μg/ml, from DIV13-DIV14) (Figure 18F-18I). These data confirm the synaptogenic properties of cholesterol and strengthen the hypothesis of the involvement of its impaired metabolism in synaptic defects observed in RTT models.

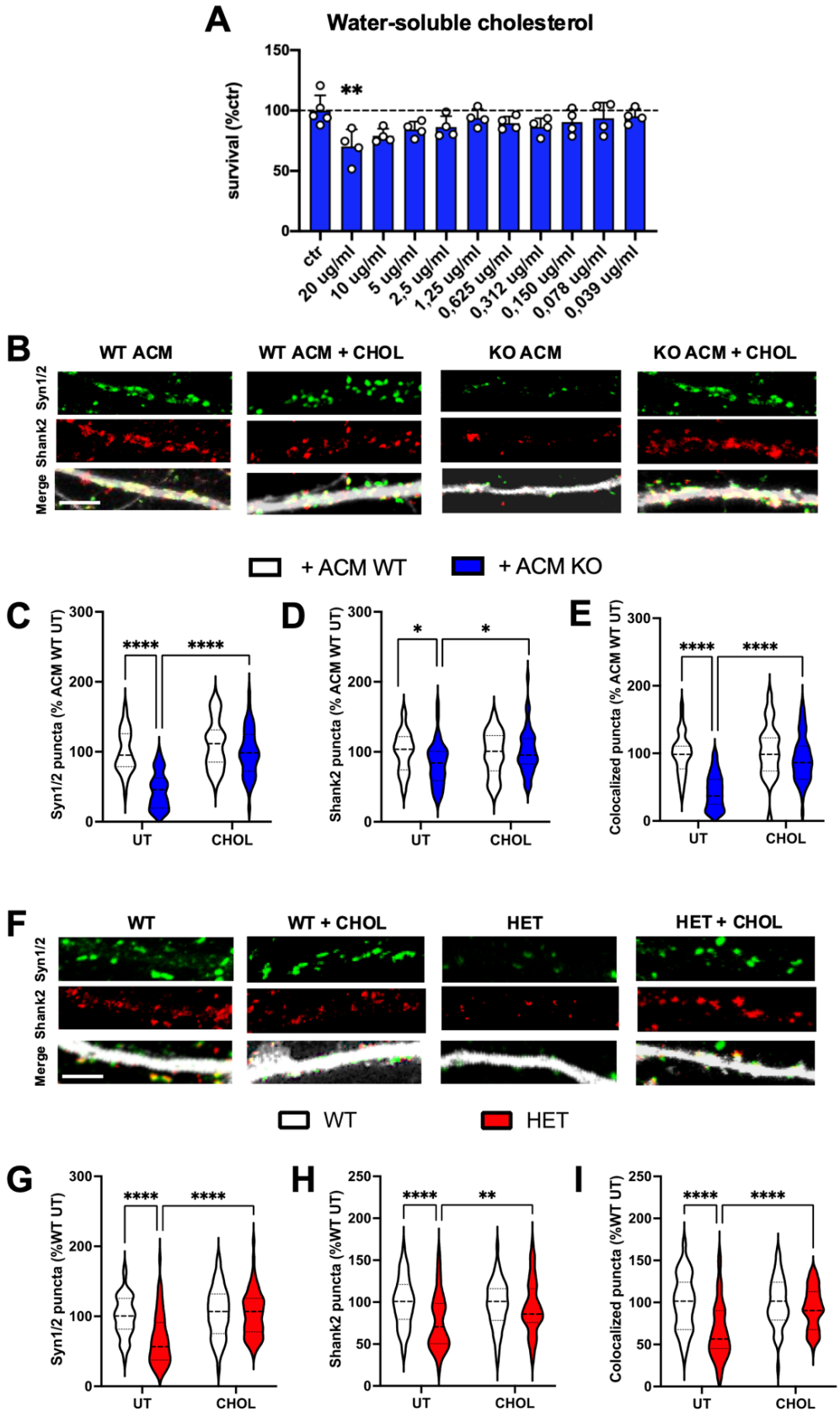

Figure 18. Water-soluble cholesterol supplementation rescues synaptic defects. **(A)** Graph represent the survival of WT cortical neurons cultured at DIV13 for 24 hours with

decreasing concentrations of a water soluble formulation of cholesterol. Data are reported as percentages with respect to untreated cells (NT) set at 100% and expressed as mean ±SEM (n=4 technical replicates). Statistical analysis is performed by one-way ANOVA test followed by Tukey's post hoc test (**p<0.01, ***p<0.001). **(B)** Representative images of primary dendrites collected from WT neurons after 24 hours WT ACM or KO ACM treatment, with or without cholesterol supplementation. Scale bar = 5 μm. Cells are stained for Synapsin1/2 (green), Shank2 (red) and MAP2 (white in merged images). **(C-E)** Violin plots indicate the median (dashed line) and 25th and 75th percentiles (dotted lines) of the number of Syn1/2 **(C)** and Shank2 **(D)** puncta and their colocalization **(E).** Values for puncta number are expressed as percentages with respect to untreated WT neurons (+ACM WT set as 100%). **(F)** Representative images of primary dendrites collected from WT and HET neurons after 24 hours treatment with or without cholesterol supplementation. Scale bar = 5 μm. Cells are stained for Syn1/2 (green), Shank2 (red) and MAP2 (white in merged images). **(G-I)** Violin plots indicate the median (dashed line) and 25th and 75th percentiles (dotted lines) of the number of Syn1/2 **(G)** and Shank2 **(H)** puncta and their colocalization **(I)**. Values for puncta number are expressed as percentages with respect to untreated WT neurons (WT UT set as 100%). For violin plots, statistical analysis is performed by two-way ANOVA followed by Tukey's post hoc test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). More than 50 neurons are analyzed for each experimental group and data are collected from at least 2 independent experiments.

4.4.4 Nsdhl expression is constantly reduced in *Mecp2* deficient models

Having observed a strong and constant downregulation of *Nsdhl* gene in KO astrocytes both in mono-culture and after MACS sorting, we investigated its expression in different RTT animal models, focusing on the most affected brain areas. This information could be instrumental for identifying a novel therapeutic target for RTT. In particular, we evaluated Nsdhl protein expression by performing western blot analysis on the cortex (CTX), hippocampus (HP) and cerebellum (CB) of both asymptomatic (P20) (Figure 19A) and symptomatic (P40) *Mecp2* KO mice (Figure 19B). We unveiled a consistent downregulation of Nsdhl in all brain areas when symptoms are not overt, that is maintained during the development of the disease. Interestingly, we found decreased Nsdhl protein level also in the CTX of symptomatic (P200) HET female animals (Figure 19C), whereas no impairment is evident in the hippocampus and cerebellum. Interestingly, Nsdhl protein level is halved in all the considered brain areas of the KI *Mecp2*$^{Y120D/y}$ (Y120D KI) mouse model, harboring a phospho-mimetic missense mutation and that phenotypically resembles the null mouse (Figure 19D).

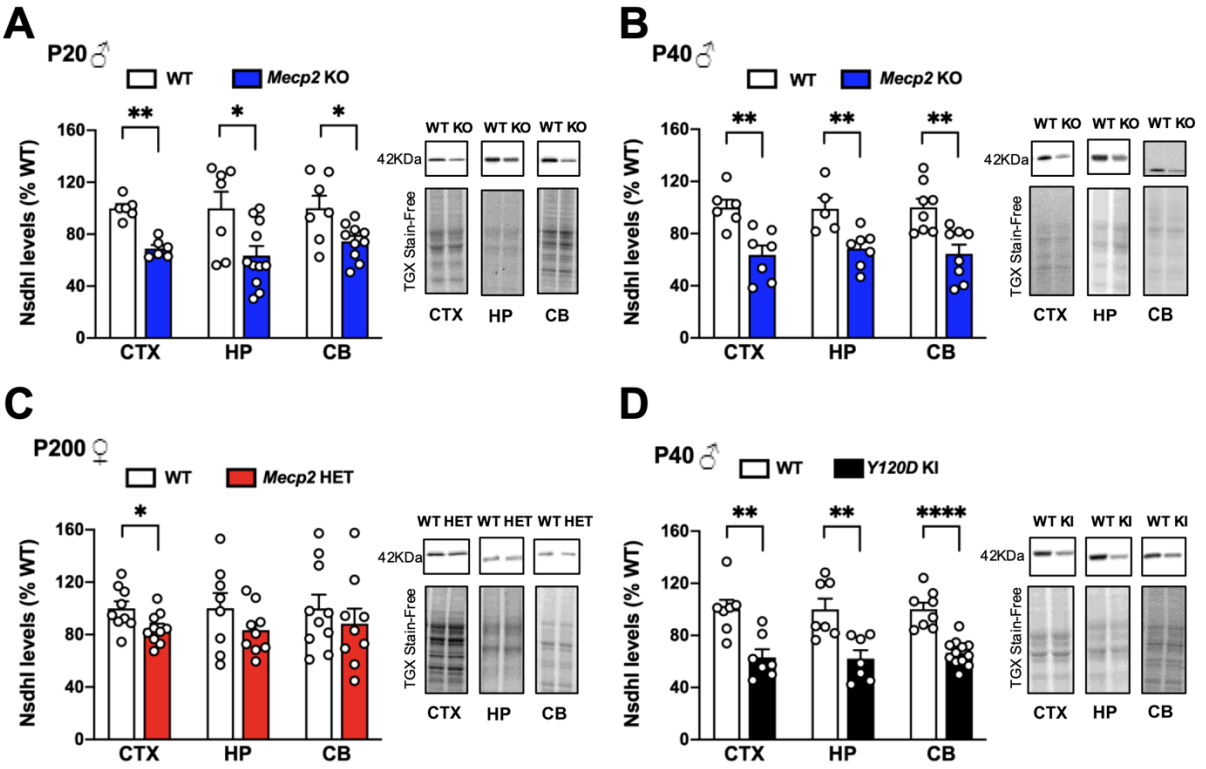

Figure 19. Nsdhl protein expression in the cortex, hippocampus and cerebellum of RTT mouse models. **(A-D)** Graphs show the protein expression level of Nsdhl (WT set at 100%) measured in the cortex (CTX), hippocampus (HP) and cerebellum (CB) of KO animals at P20 **(A)** and P40 **(B)**, of P200 HET females **(C)** and P40 *Mecp2*$^{Y120D/y}$ KI male animals **(D)**. The experiment was conducted on at least 5 samples for each experimental group. Statistical analysis was performed by Student's t-test (*p<0.05, **p<0.01, ****p<0.0001). Bars represent the mean ± SEM. For each graph, on the right, representative images of Nsdhl protein (42 KDa) in the selected brain areas are shown. Nsdhl band intensity is normalized on the total protein content of the sample measured on TGX Stain-Free gel.

5. Discussion

Defects in neuronal morphology, activity and synaptic transmission are an hallmark of Rett syndrome and they have been extensively characterized (59,61,62,81,83). First evidence of the contribution of astrocytes in determining neuronal defects in RTT came out more recently. Indeed, both mouse and human *Mecp2* deficient astrocytes not only show alterations of process branching, metabolism and calcium waves (104,147,149,150), but they are not able to properly support neuronal growth and activity (103,151,152). In this context, we investigated the less explored impact of *Mecp2* KO astrocytes on synaptic phenotype and, as astrocytes elicit many of their functions by releasing gliotransmitters, we particularly focused on the study of secreted paracrine signals (112,135,137,184). For this purpose, we used two different *in vitro* culture systems: on one hand, to consider the factors secreted by astrocytes only in dependence of their genotype, we treated neurons with astrocyte conditioned medium (ACM) obtained from mono-cultures of WT or *Mecp2* KO astrocytes. On the other hand, we exploited a co-culture system between neurons and astrocytes, in which the latter are cultured on transwell inserts, which prevent the direct contact with neurons while allowing the exchange of secreted cues. By immunofluorescence staining of pre- and post-synaptic proteins, we found that the synaptic phenotype is severely compromised in both the *in vitro* systems. This evidence suggests that *Mecp2* KO astrocytes are influencing synaptogenesis and synaptic maintenance by releasing synaptotoxic molecules or by failing to secrete sufficient synaptogenic factors. Most likely, a synergic cooperation between these two aspects might contribute to the observed defects and, in this work, we describe the involvement of two different astrocyte-secreted molecules and their effects on synapses.

5.1 Mecp2 KO astrocytes affect synaptogenesis by Interleukin-6 dependent mechanisms.

After the first evidence of the negative impact of *Mecp2* deficient astrocytes on neuronal phenotypes, many researches focused on the investigation of astrocyte secretome to identify detrimental targetable factors and develop new therapeutic strategies (150,153,163). However, despite many putative molecules have been identified, presented studies only included the investigation of proteins and metabolites

released from astrocytes cultured alone. A key strength of our work is the investigation of astrocyte-neuron crosstalk with a focus on the molecules secreted by astrocytes in presence of neurons. Undeniably, neurons deeply influence astrocyte morphology, gene expression and functioning (109,138,139) and the transwell-based system enabled us to investigate this aspect in a more physiological and comprehensive manner. To investigate the nature of molecules that could influence synaptic function, we performed a bulk RNA-sequencing to compare gene expression in WT neurons cultured alone or in presence of either WT or *Mecp2* KO astrocytes seeded on transwells. Our aim was to unveil downstream deregulated pathways affecting neuronal health and infer the upstream molecules involved. The choice of this indirect approach, instead of a more direct proteomic analyses in the culture medium, was essentially due to the challenges related to the presence of serum in the co-culture medium: high levels of albumin hinder the detection of low-abundant proteins and favor the formation of complexes with other factors, affecting their identification. The reliability of the selected transcriptomic methodology has been demonstrated by the fact that Gene Ontology (GO) analysis highlights a downregulations of the genes related to synaptic assembly and transmission in neurons cultured with *Mecp2* KO astrocytes, confirming the strong alteration of synaptic phenotype showed by immunofluorescence staining in the same experimental condition. Moreover, in line with literature, the co-culture condition, regardless of *Mecp2* presence in astrocytes, promotes the expression of several genes involved in neuronal maturation, confirming the ability of astrocyte to promote neuronal growth (164,185) . Notably, our findings also revealed that molecules secreted by KO astrocytes induce an abnormal inflammatory response in neurons; to this end it is well known that inflammation affects synaptic organization and function in the developing brain (186,187). Indeed, mental disorders are frequently characterized by neuroinflammatory components and, in the context of RTT, extensive data describe a chronic, sub-clinical inflammation in patients (96,188–190). Importantly, MeCP2 plays a role in regulating the expression of genes related to inflammation across various cell types, as microglia and macrophages, resulting in an aberrant NF-κB pathway activation and alteration in cytokines and chemokines production (94,191,192). Therefore, we quantified the amount of a specific set of cytokines in co-culture medium exploiting Luminex technology, unveiling a significant increase of IL-6 concentration when neurons are co-cultured with *Mecp2* KO astrocytes. Of note, while we found a significantly higher IL-6 expression in *Mecp2*

KO astrocytes cultured on transwell, its level remains very low in co-cultured neurons, indicating astrocytes as primary source of the cytokine.

The relevance of IL-6 and its pathway for mental diseases is extensively described in literature. This pleiotropic cytokine is involved in the regulation of neuronal development and survival. Moreover it regulates synaptic formation and functioning (193–195). Interestingly, according to its concentration and brain region, IL-6 can exert completely opposing activities, either synaptogenic or synaptotoxic. For instance, the elevation of IL-6 level is reported to promote glutamatergic synaptogenesis, while significantly decreasing the number of inhibitory synapses in hippocampus, thus altering the excitatory/inhibitory balance (196–198). In general, IL-6 overexpression in the mouse brain often correlates with neurological abnormalities observed in a plethora of brain disorders, including Alzheimer disease (AD), Parkinson disease (PD), multiple sclerosis (MS), schizophrenia and autism (199–203). Additionally, it is worth to mention that IL-6 increased expression has already been described in the brain, saliva, and plasma of patients suffering from RTT (191,204).

We elucidated the impact of IL-6 overproduction by *Mecp2* KO astrocytes on neuronal health, and particularly on synapses, by preventing its activity with the administration in the co-cultures of a neutralizing antibody able to bind the cytokine with picomolar affinity. As previously described, neurons co-cultured with *Mecp2* KO astrocytes for 14 days display significantly reduced dendritic length, in line with literature, and significant defects in pre- and post-synaptic protein density. Treatment with neutralizing antibody is sufficient to rescue neuronal morphology, pre-synaptic phenotype and significantly ameliorates the defect at post-synaptic compartment. However, the persistence of the post-synaptic defect suggest that other molecules beside IL-6 could elicit a synaptotoxic activity or the lack of beneficial cues is still impacting on synapses. For instance, analyzing the expression of a set of genes coding for proteins involved in inflammatory response in astrocytes on transwells, we found that *Mecp2* KO astrocytes show a strong increase also in the level of other cytokines as *IL-1β* and *Cxcl12*, that may exacerbate the synaptic defect. The treatment with neutralizing antibody also underlined the importance of IL-6 pathway in physiological conditions, since blocking the activity of the cytokine in neurons cultured with WT astrocytes is detrimental for pre-synaptic compartment, demonstrating that the cytokine is required for synaptogenesis and synaptic maintenance and that a fine tuning of its concentration is necessary to maintain neuronal homeostasis. Further confirming the synaptotoxic

action of IL-6, a chronic administration of the cytokine on WT cortical neurons negatively affects synaptic phenotype, clearly demonstrating the detrimental effects of IL-6 on synapses and highlighting this cytokine as a potential therapeutic target for RTT. Notably, IL-6 and its pathway have already been proposed for the development of novel therapies. As an example, modulation of IL-6 signaling in brain of BTBR mice, used as a reliable model for autism-relevant features, is sufficient to ameliorate animal social behaviors (205). Espinal and colleagues found that treatment of neurons with ACM deriving from PD astrocytes cause neurodegeneration, that is effectively prevented treating astrocytes with a FDA-approved neutralizing antibody for IL-6, Tocilizumab (200). For this reason, in the next future, we aim to test the *in vivo* efficacy of a neutralizing antibody in RTT mice in ameliorating molecular and behavioral phenotypes. Importantly, the therapy should aim at attenuating IL-6 activity without completely abolishing it, considering its aforementioned physiological roles. A possibility to maintain IL-6 beneficial effects after treatment stems from the fact that the cytokine acts through two different signaling pathways. The “classical” one involves the binding of IL-6 to the membrane-bound IL-6 receptor α-subunit (mIL-6R) and glycoprotein 130 (gp130) signal-transducing subunit. This signaling seems to mainly mediate the anti-inflammatory activities of IL-6. However, while gp130 is expressed on all cell types, mIL-6R is only present on few cells in the body (hepatocytes and some leukocytes), but a soluble form of IL-6R (sIL-6R) can bind IL-6 with a similar affinity as the mIL-6R. This complex can bind to gp130 in a process named “trans-signaling”. Many antibodies have been developed to block both the pathways, but in the past decade, trans-signaling has emerged as the predominant by which IL-6 promotes disease pathogenesis. Indeed, trans-signaling mediates the pro-inflammatory activity of the cytokine (206,207). Selective inhibitors of IL-6 trans-signaling have shown therapeutic potential in various preclinical models of inflammatory-related diseases and we are considering to exploit these drugs to achieve a safer and more translational treatment in our mouse model.

A novel and interesting aspect of this works relies on the evidence that the secretome of *Mecp2* KO astrocytes is influenced by the genotype of the neighboring neurons, underlining the importance of non-cell autonomous communication mechanisms in RTT. In particular, we first highlighted the increase of IL-6 mRNA level in KO astrocytes only when maintained in presence of WT neurons and the secretion of the cytokine declines when neurons are removed. Accordingly, IL-6 level is not increased in KO

astrocytes cultured alone. These molecular findings underscore the critical role of neurons in modulating astrocyte function and gliotransmitter release. Notably, we observed that the increased expression of IL-6 occurs specifically in KO astrocytes when co-cultured with WT neurons, but not in presence of KO neurons. The phenomenon underlying our evidence is called “cell interference hypothesis" and has been proposed for other diseases, such as for PCDH19 epilepsy, an X linked disorder in which males with a hemizygous mutation in protocadherin-19 gene show less disruptive symptoms than the females carrying the mosaic mutations (208). This theory states that in certain genetic disorders characterized by mosaicism, the presence of both mutant and normal cells within the same organism can lead to abnormal cellular interactions that exacerbate the disease phenotype. In our model the molecular mechanisms generating this selective regulation remain unknown but it might be due to neuronal-derived signals, such as neurotransmitters and neuromodulators, potentially associated with neuronal activity.

One of the mostly relevant aspects of astrocyte-neuron crosstalk that is not embraced in our work is the role of physical interactions between these two cell types, particularly for the formation and regulation of the tripartite synapse structure (209). Actually, our preliminary data show that, even if secreted factors released by *Mecp2* KO astrocytes are sufficient to reduce pre- and post-synaptic puncta density, the physical contact of neurons with *Mecp2* deficient astrocytes also significantly impacts on the area of pre-synaptic protein clusters, worsening the synaptic defect. Indeed, further studies on in contact co-culture systems could unveil other molecules expressed by RTT astrocytes contributing to the impairment of synapses. As an hypothesis, basing on literature, the presence of more pronounced synaptic defects may be determined by the involvement of cell adhesion molecules (210,211). Despite the described limitation, we were able to observe the deregulation of IL-6 also in more comprehensive and physiological RTT models. For this purpose, we characterized IL-6 pathway in the *Mecp2*$^{+/-}$ (HET) females, since they better represent the condition of RTT patients. In fact, since they are heterozygous for the mutated allele, they show different grades of mosaicism due to skewed X chromosome inactivation, resulting in a huge variability of symptoms manifestation. The coexistence of cells expressing a WT copy of *Mecp2* (*Mecp2*+) and *Mecp2* deficient (*Mecp2*-) cells in HET brains is particularly relevant for this study. Indeed, as before outlined, the fact that astrocyte secretome is influenced by the genotype of neighboring cells suggests that the brain of a full *Mecp2* KO mouse could

differ significantly from the one of a HET female mouse. Interestingly, we reported a significant increase of IL-6 gene expression in astrocytes sorted with MACS technology from asymptomatic P7 HET mice, corroborating the presence of the defect also in astrocytes developed in the context of a mosaic brain and without the presence of serum, a condition that could impact astrocytes physiology. Interestingly, we also reported an inverse correlation between *Mecp2* and *IL-6* expression, further reinforcing the link between Mecp2 deficiency and induction of inflammatory phenotypes. A strong tendency to the upregulation of IL-6 is also observed in the cortex of symptomatic P100 and P200 HET female mice, demonstrating that the inflammatory trigger persists during disease progression. However, our data indicate that the Stat3 phosphorylation, the major target of the binding of IL-6 to its receptor, does not change between WT and HET cortices. This result might depend on the selective deregulation of IL-6 occurring in astrocytes, since the analysis in the whole tissues could dilute the defects. For this reason, we aim to directly analyze the protein levels of the cytokine in astrocytes from WT and HET mice cortices at P100. Moreover, it could be interesting to improve our knowledge on the mechanism underlying IL-6 overexpression by separating *Mecp2*+ and *Mecp2*- astrocytes populations in HET brains and investigating IL-6 pathway in these two experimental groups.

To conclude, this part of our study unveiled the synaptotoxic effect of excessive release of IL-6 by *Mecp2* KO astrocytes, providing a promising target for the development of novel therapies for RTT and encouraging studies on this pathway in other neurodevelopmental disorders. Nevertheless, certainly, IL-6 is not the only molecule causing synaptic alterations in RTT. Indeed, as shown in preliminary data, even 24h KO ACM treatment is sufficient to induce a synaptic defect. Our findings revealed the synaptotoxic nature of certain factors, which may include cytokines, enzymes, or hormones. Moreover, notably, we propose the existence of a synergistic interaction between the increased release of toxic molecules and the diminished secretion of synaptogenic factors.

5.2 Deregulation of cholesterol pathway in Mecp2 KO astrocytes contributes to synaptic defects.

After unveiling the synaptotoxic effect of IL-6 secretion on neurons, literature evidence led us to focus on the investigation of one of the first studied synaptogenic molecules,

such as cholesterol. All cholesterol in the CNS is produced locally, since blood–brain barrier (BBB) prevents the entry of systemic cholesterol-rich lipoproteins. In early development, before BBB closure, neurons are able to produce cholesterol to support the rapid growth and formation of synapses. In adult brain, whereas oligodendrocytes rely on their own cholesterol synthesis to form myelin, neurons, which need large amounts of this lipid to maintain their morphology and intense activity, mainly import cholesterol from astrocytes (212). Cholesterol released by astrocytes is known to exert a crucial role in neuronal maturation and synaptogenesis, as suggested by dendritic spines and synapses degeneration resulting from its depletion (213–215). This lipid increases the formation of synaptic vesicles at the pre-synaptic terminal and stabilizes the level of NMDARs receptors at post-synaptic side, enhancing neuronal activity (133,216–218). Several researches report an alteration of brain cholesterol metabolism in several neurological disorders, as Fragile X syndrome (FXS), AD, PD and Huntington's diseases (HD) (214,219–221). For what concerns RTT, a reduction in the rate of cholesterol biosynthesis has been reported in the brain of *Mecp2* KO mice. Moreover, researchers found a substantial downregulation in the expression of key enzymes for its metabolism and in the concentration of cholesterol and its precursors (100,173–175). Furthermore, more recently, a reduction in cholesterol in the CSF of RTT patients was detected (176). In this context, the novelty of our study relies on the investigation of cholesterol pathway with a focus on astrocytes, that constitute the unique source in the adult CNS of this lipid for neurons. We started our investigation from the simplest *in vitro* condition, represented by WT and *Mecp2* KO astrocytes mono-cultures, looking at the expression level of a panel of genes coding for proteins involved in cholesterol metabolism and transport, in order to gain insight into different sides of this complex mechanism. We found a consistent downregulation of most of these genes in *Mecp2* KO astrocytes, suggesting both an altered cholesterol production and delivery. Conversely, the level of expression of ApoE, the principal carrier of cholesterol to neurons is not affected. To gain insight on the putative involvement of cholesterol metabolism in a more comprehensive RTT model, we evaluated the expression of the same genes in astrocytes isolated by MACS sorting from P7 mice cortices, highlighting a decrease in the level of *Hmgcr* and *Nsdhl* in *Mecp2* KO cells. Actually, some of the genes that are downregulated in *Mecp2* KO astrocytes in mono-cultures are not differentially expressed in sorted astrocytes, confirming how gene expression in astrocytes is deeply influenced by the interactions

with other cells. However, importantly, *Hmgcr* and *Nsdhl* are really crucial genes for brain homeostasis maintenance, as demonstrated by the devastating disorders originating from their dysfunction (222,223). Observed downregulation of genes related to cholesterol metabolism suggest an impairment in cholesterol production. For this reason, we are currently quantifying the amount of cholesterol released by WT and *Mecp2* KO astrocytes in culture medium, in order to assess the effective functioning of the pathway and the amount of cholesterol that could be able to reach neurons after secretion. To achieve a sufficiently sensitive measurement, we started quantifying the cholesterol released in ACM with the Amplex Red Cholesterol Assay Kit, but we were not able to unveil differences between WT and KO ACM. We hypothesized that this result could be influenced from the indirect nature of the biochemical assay, that operates through a series of enzymatic reactions ultimately generating a fluorescent signal proportional to the cholesterol concentration. Therefore, we attempted to quantify cholesterol using a direct assay, such as thin-layer chromatography. However, this approach lacked the sensitivity required to detect subtle differences between samples. So, currently, we are exploiting gas-chromatography, that is sufficiently sensitive to accurately measure cholesterol amount in ACM.

While characterizing this important aspect, we tested whether cholesterol supplementation could ameliorate the occurrence of synaptic defects induced in neurons by KO ACM. Encouragingly, cholesterol addition was sufficient to rescue the number of both pre- and post-synaptic puncta. Of relevance for RTT, we observed a beneficial effect of cholesterol addition also on cortical neurons derived from HET embryos, that are known to show severe synaptic alterations, as the ones described in *Mecp2* KO neurons (224). These experiments represent an additional indication of the involvement of cholesterol metabolism alterations in synaptic phenotypes observed in RTT and open up the possibility to consider cholesterol administration as a treatment strategy. Notably, intraperitoneal injections of cholesterol loaded nanoparticles has been already proven to be efficacious in the treatment of motor and cognitive defects in an animal model of Huntington's disease (214). However, high cholesterol levels have been detected in the serum of RTT patients (173), indicating a different regulation of peripheral and brain cholesterol. Therefore, a specific targeting of brain, that could be achieved, for instance, by intranasal administration, would be useful to avoid systemic complications (225). Alternatively to cholesterol supplementation, a rescue of defective metabolism could be obtain by modulating the complex pathway related to

this lipid. For this purpose, we decided to treat RTT astrocytes mono-cultures with Trofinetide, the only FDA-approved drug for RTT, that has been reported to affect metabolism among many other cellular processes (179,180). We found relevant to test if this drug could affect the expression of cholesterol-related genes. Intriguingly, the significant reduction of the expression of *Abca1*, *Mvk* and *Abcg1* genes, involved in cholesterol synthesis and transport, reported in *Mecp2* KO astrocytes is no more present after treatment. This data suggest that part of the beneficial effect elicited by Trofinetide on RTT patients may be due to the modulation of cholesterol metabolism. Another possible treatment strategy relies in correction of the expression of genes associated to cholesterol synthesis and release by gene therapy. To this regard, gene therapy for *Srebp2* (coding for the transcription factor regulating the expression of most of cholesterol genes) demonstrated to be effective in animal models of HD whereas expression of *Cyp46a1* gene (coding from an enzyme serving as primary strategy to eliminate cholesterol in the brain) improve symptoms of HD and Niemann-Pick disease (226–228). Very recently, the overexpression of *Cyp46a1* in neurons has been also tested for the treatment of RTT, with encouraging results obtained in both *Mecp2* KO male and HET female animals (229). In this work, we identified *NSDHL* as an interesting target gene in RTT. This gene codes for NAD(P)-dependent steroid dehydrogenase-like protein, an essential enzyme for cholesterol biosynthesis pathway. *NSDHL* mutations causes CHILD (Congenital Hemidysplasia with Ichthyosiform nevus and Limb Defects) syndrome, a X-linked dominant disorder of lipid metabolism with disturbed cholesterol biosynthesis and typically lethal in males (230). We observed that transcriptional expression of *Nsdhl* is consistently and significantly downregulated in all our RNA-sequencing datasets, spanning from *in vitro* cultures of *Mecp2* KO neurons derived from neuronal precursor cells and *Mecp2* KO primary cortical neurons, to *ex vivo* cortical and hippocampal tissues of symptomatic *Mecp2* KO mice. Moreover, *Nsdhl* downregulation is confirmed in RNA-sequencing data from both humans and animal models (101,231–233). Interestingly, *NSDHL* expression shows an opposite trend in *MECP2* duplication syndrome, indicating a direct correlation between *MECP2* expression and *NSDHL* transcription (63,234,235). In support of this association, the intravenous delivery of *Mecp2* transgene in a *Mecp2* KO mouse rescued the expression levels of *Nsdhl* and other crucial enzymes of the same pathway (231) and, very recently, CUT and Tag analysis revealed that the expression of *NSDHL* is controlled by the association of MeCP2 with Polymerase II (236). For what regards

Nsdhl protein expression, we found it significantly reduced in all brain areas of both asymptomatic and symptomatic *Mecp2* KO mice, demonstrating that the defect is already present when symptoms are not overt and is maintained during disease progression. Despite Nsdhl protein level is significantly decreased also in the cortex of symptomatic HET females, the reduction is less pronounced, with no differential expression in cerebellum and hippocampus, probably due to mosaicism. Conversely, there is a consistent downregulation of the protein in all the brain areas of Y120D KI mice, which are phenotypically very similar to KO mice, further demonstrating that impaired functioning of *Mecp2* strongly affects *Nsdhl* expression. Therefore, this study opens up the possibility of testing a gene therapy strategy directed to the re-expression of *Nsdhl* in *Mecp2* KO mice, to better understand the involvement of this protein in disease pathogenesis.

5.3 Conclusion

This study reveals that the overproduction of IL-6 and the impairment of cholesterol metabolism in *Mecp2* KO astrocytes have detrimental effects on synaptic health. Indeed, these findings support the hypothesis that insufficient release of synaptogenic factors by astrocytes, combined with the release of synaptotoxic cues, contributes to pre- and post-synaptic impairments characterizing the pathology. It is worth to notice that the two pathways we found deregulated in mutant astrocytes are very different, since one is associated to inflammatory processes and the other to lipid metabolism. These results underscore the complexity of the pathogenesis of RTT, that probably derives from the multifunctional nature of MeCP2 and its critical role in regulating thousands of genes. Indeed, mutations in *MECP2* gene lead to the deregulation of several cellular mechanisms posing a challenge in identifying key causative pathways in RTT. Our data unveiled two potential molecular targets for testing novel treatment strategies, demonstrating that a deep understanding of the dysfunctional processes involved in the disease pathogenesis is crucial. Finally, from a translational perspective, we propose that testing a combination of drugs targeting different pathways could be beneficial for the improvement of the quality of life for RTT patients.

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- Table 1: *PCR cycles for genotyping and sex determination*.
- Table 3: List of the genes tested by qRT-PCR and their respective primers.

Appendix

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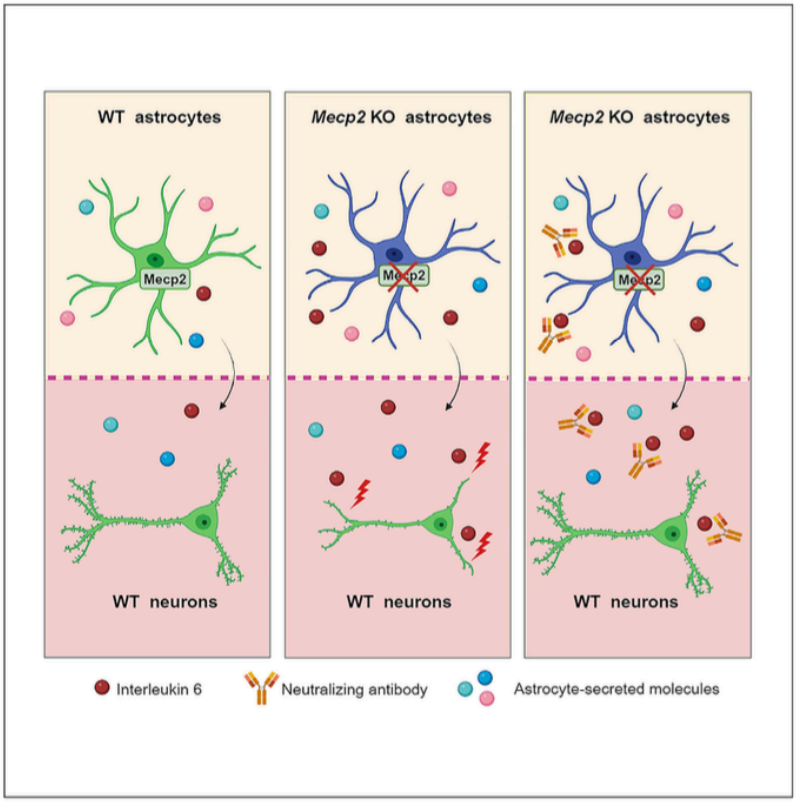
Mecp2 knock-out astrocytes affect synaptogenesis by interleukin 6 dependent mechanisms



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Highlights

High levels of IL-6 secreted by *Mecp2* KO astrocytes negatively affect synapses

IL-6 upregulation results from an altered astrocyte-neuron crosstalk in Rett syndrome

Astrocytes from *Mecp2* heterozygous mouse cortices express high levels of IL-6

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Mecp2 knock-out astrocytes affect synaptogenesis by interleukin 6 dependent mechanisms

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SUMMARY

Synaptic abnormalities are a hallmark of several neurological diseases, and clarification of the underlying mechanisms represents a crucial step toward the development of therapeutic strategies. Rett syndrome (RTT) is a rare neurodevelopmental disorder, mainly affecting females, caused by mutations in the X-linked methyl-CpG-binding protein 2 (*MECP2*) gene, leading to a deep derangement of synaptic connectivity. Although initial studies supported the exclusive involvement of neurons, recent data have highlighted the pivotal contribution of astrocytes in RTT pathogenesis through non-cell autonomous mechanisms. Since astrocytes regulate synapse formation and functionality by releasing multiple molecules, we investigated the influence of soluble factors secreted by *Mecp2* knock-out (KO) astrocytes on synapses. We found that *Mecp2* deficiency in astrocytes negatively affects their ability to support synaptogenesis by releasing synaptotoxic molecules. Notably, neuronal inputs from a dysfunctional astrocyte-neuron crosstalk lead KO astrocytes to aberrantly express IL-6, and blocking IL-6 activity prevents synaptic alterations.

INTRODUCTION

Astrocytes represent one of the most abundant classes of glial cells in the mammalian brain and play a pivotal role for proper health and function of the central nervous system (CNS), providing metabolic and trophic support to neurons.[1] Accordingly, astrocyte-neuron interactions are fundamental for synaptic development across different brain regions since early embryonic stages to adulthood.[2,3] By secreting bioactive proteins, such as neurotrophins, synaptogenic molecules, cytokines, and chemokines, astrocytes finely promote synaptic formation, functional maturation, and refinement.[3–6] Consequently, altered secretion of these signals contributes to synaptic defects, as described for many neurodevelopmental, neuropsychiatric, and neurodegenerative disorders.[7–10] Rett syndrome (RTT) is a rare neurodevelopmental disease caused in the vast majority of cases by mutations in the X-linked methyl-CpG-binding protein 2 (*MECP2*).[11] Besides neurons, which suffer from severe morphological, functional, and synaptic defects,[12–15] recent data indicated a role for astrocytes in RTT pathogenesis, reporting that *Mecp2* mutant astrocytes and their conditioned medium exert a negative effect on neuronal development, decreasing dendritic outgrowth and affecting overall maturation.[16–19] The defective neuronal support by *MECP2* mutant astrocytes might be the result of several alterations, including molecular, morphological, metabolic, and functional defects.[19–21] Accordingly, the sole loss of *Mecp2* in astrocytes is sufficient to cause pathological alterations, while rescuing *Mecp2* expression specifically in these glial cells greatly ameliorates RTT symptoms and leads to synaptic improvements, suggesting the possibility to target astrocytes for therapeutic purpose.[17] Two proteomic studies analyzed deregulated proteins secreted by *Mecp2* knock-out (KO) astrocytes to disclose molecules influencing dendritic maturation. Ehinger and colleagues examined the secretome of KO cortical astrocytes isolated from P1-2 pups, revealing the decrease of Lcn2 and Lgals3, that, when added to null neurons, effectively improved dendritic arborization.[22] More recently, Caldwell and collaborators analyzed the proteome of culture medium from KO cortical astrocytes isolated at P7 by immunopanning, reporting the alteration of several proteins, including an increase of Igfbp2 and BMP6, that were highlighted as molecular candidates involved in the occurrence of neuronal defects.[23] However, the aforementioned studies analyzed the secretome of astrocytes cultured alone, without considering their crosstalk with neurons, which strongly affects molecular and functional properties of both cell populations,[24–26] an issue particularly relevant for RTT, which is characterized

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by mosaic interactions between cells expressing either the wild type (WT) or mutant *MECP2* allele. Thus, in our study, we explored whether and how *Mecp2* KO astrocytes might alter synapses in WT neurons, investigating the astrocyte-neuron crosstalk in a co-culture system. We report that KO astrocytes secrete molecules that alter synaptogenesis in WT neurons and transcriptomic analyses suggested the involvement of inflammatory triggers. Accordingly, molecular investigations on KO astrocytes revealed an increase of interleukin-6 (IL-6) levels in the co-culture medium (CCM) as well as of its transcription, and we proved the causative role of this cytokine in synaptic impairments. Interestingly, excessive secretion of IL-6 exclusively occurs in the presence of WT neurons and lacks when KO astrocytes are cultured alone.

Overall, our study identifies a novel pathogenic mechanism triggered by the communication between WT neurons and *Mecp2* KO astrocytes that leads to synaptic alterations and might provide a novel therapeutic target for RTT and other *MECP2*-related disorders.

RESULTS

Molecules secreted by *Mecp2* KO astrocytes affect synaptogenesis in WT neurons

Since astrocytes play key roles in synapse formation and RTT pathogenesis,[3,16,17] we exploited a transwell-based co-culture between WT cortical neurons and *Mecp2* KO astrocytes to investigate the consequence of *Mecp2* deficiency in astrocytes on synaptogenesis. WT-WT co-cultures were used as control. The impact on synaptogenesis was assessed by analyzing the density of pre- and post-synaptic puncta, and their colocalization, as an index of functional synapses (Figure 1A). WT neurons matured under the influence of KO cortical astrocytes displayed reduced number of both pre- and post-synaptic puncta as well as of colocalized puncta, compared to control (Figures 1B and 1C), with no change in size (Figure S1). Of note, KO astrocytes cultured in close contact with WT neurons caused reduction of pre-synaptic puncta density, as well as of puncta area (Figure S2), thus suggesting that other factors beyond secreted molecules might alter the synaptic phenotype.

According to the heterogeneous properties of astrocytes depending on cerebral region, we assessed whether *Mecp2* deficiency diversely affects their synaptogenic potential when derived from brain regions other than the cortex.[27] Interestingly, WT cortical neurons cultured with hippocampal KO astrocytes exhibited a selective impairment of the density of pre-synaptic puncta, with no effect on post-synapses and co-localization (Figures 1D and 1E), while cerebellar astrocytes did not cause any synaptic defect (Figures 1F and 1G).

In line with the morphological analysis, electrophysiological recording of excitatory synaptic basal transmission showed that neurons co-cultured with KO cortical astrocytes display a significant reduction of miniature excitatory post-synaptic currents' (mEPSCs) frequency if compared to neurons with WT astrocytes. To note, a slight significant increase of mEPSC amplitude was observed in neurons with KO astrocytes, whereas the passive properties were not changed (Figures 1H, 1I, and S3).

These results demonstrate that molecules secreted by *Mecp2* KO cortical astrocytes impair synaptogenesis, therefore supporting a non-cell autonomous influence on WT neurons. The release of neurotoxic factors and/or defective secretion of synaptogenic molecules might be involved. Moreover, this effect is brain-area specific, as astrocytes from other brain areas, including the hippocampus and cerebellum, did not show the same capacity to influence synaptogenesis, pointing to cortical astrocytes as the most affected cells.

Transcriptional profile of WT neurons confirms the detrimental action of *Mecp2* KO astrocytes on synapse formation, highlighting the role of pro-inflammatory cues

To gain insights into the effects mediated by cortical astrocytes on neurons, we performed bulk RNA-seq on WT neurons cultured with astrocytes, which were plated on transwell inserts (Figure 2). Transcriptomic analysis was conducted on neurons derived from three experimental groups, in which neuronal cells were cultured alone ("CTRL"), with WT astrocytes ("+aWT"), or with *Mecp2* KO astrocytes ("+aKO") (Figure 2A). Transcriptional profiles of +aWT or +aKO neurons were compared to each other (+aKO versus +aWT) and to that of CTRL (+aWT versus CTRL; +aKO versus CTRL). Principal component analysis (PCA) of whole transcriptome expression highlighted that neurons from co-culture systems clustered together with respect to CTRL (Figure S4A), confirming that neurons dramatically change their transcriptional profiles when cultured with astrocytes.[24] Indeed, both +aWT and +aKO showed a great number of differentially expressed genes (DEGs) when compared to CTRL, whereas few significant DEGs emerged from their comparison (Figures 2B and S4B; Tables S1–S3).

In order to identify the main biological processes influenced by the different conditions, gene ontology (GO) enrichment analysis was performed separately on downregulated or upregulated DEGs. Considering downregulated DEGs, pathways related to axonogenesis, synapse organization, dendritic development, and cognition emerged as the most affected in the comparison between +aKO versus CTRL (Figure 2C). Interestingly, the same pathways were not present in the comparison +aWT versus CTRL (Figure 2D), suggesting that KO astrocytes release synaptotoxic paracrine signals.

Notably, although a comparison between downregulated DEGs of +aKO versus +aWT was possible only using a padj < 0.1, processes related to synapse organization and activity appeared as the most affected ones (Figure S4C; Table S4). Collectively, these results highlighted the impact of *Mecp2* deficient astrocytes on proper synaptogenesis and neuronal maturation, thereby validating the immunofluorescence data.

To better investigate the overall molecular pathways affected in WT neurons by KO astrocytes, a preranked gene set enrichment analysis (GSEA) was conducted on the comparison +aKO versus +aWT. The analysis yielded several negatively correlated gene sets and only few positively correlated gene sets (NES<0 or >0, respectively, combined with FDR q < 0.05, Table S6). Among the top ten negatively correlated gene sets, we found "*glutamate receptor signaling pathway*," "*protein localization to synapse*," and "*regulation of synaptic plasticity*," thus corroborating the morphological and functional alterations observed at synaptic level (Figure 2E).

Since the direct comparison of +aWT vs. +aKO did not provide any mechanistic suggestion for the onset of synaptic alterations, we focused on the comparison of +aWT vs. CTRL and +aKO vs. CTRL, by exploring the upregulated pathways derived from the GO analyses

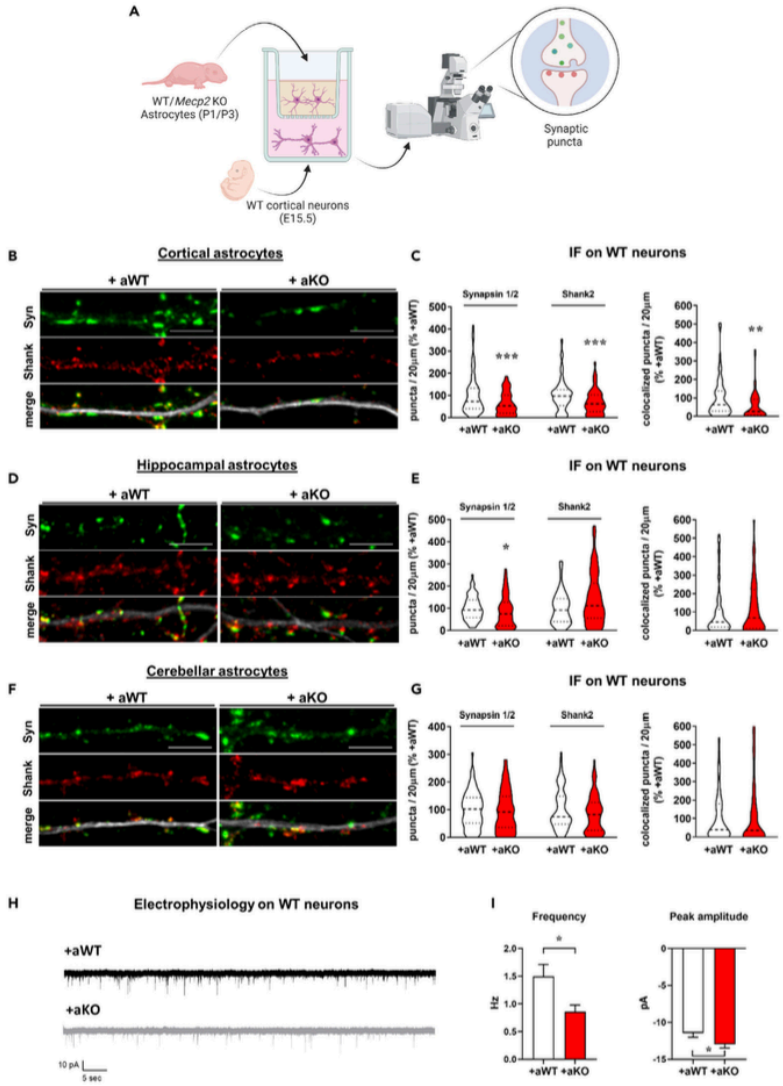

Figure 1. Soluble factors secreted by *Mecp2* KO astrocytes affect synaptogenesis, with cortical astrocytes showing the most detrimental effects

(A) Experimental design overview.

(B, D, and F) Representative images of primary branches from WT neurons (DIV14) immunostained for Synapsin1/2 (green), Shank2 (red) and their merge with MAP2 (white), in co-culture with cortical (B), hippocampal (D) and cerebellar astrocytes (F). Scale bar = 5 µm.

(C, E, and G) Violin plots indicate the median (dashed line) and 25th and 75th percentiles (dotted lines) of Synapsin1/2, Shank2 and colocalized puncta density. Values for puncta number are expressed as percentages compared to WT-WT co-cultures (set at 100%). *p < 0.05, **p < 0.01, ***p < 0.001 by Mann-Whitney test. Analyses were performed on n > 60 neurons (in C), on n > 66 neurons (in E) and on n > 46 neurons (in G) per experimental group from N > 7 (in C and E) or N > 5 (in G) biological replicates. All data derived from at least 2 independent experiments.

(H) Representative traces of miniature excitatory post-synaptic currents (mEPSCs) recorded in neurons cultured with either WT or KO astrocytes.

(I) Quantitative analysis of frequency and amplitude of mEPSCs. Data are represented as mean ± SEM. *p < 0.05 by Student's t test. WT n = 27; KO n = 35.

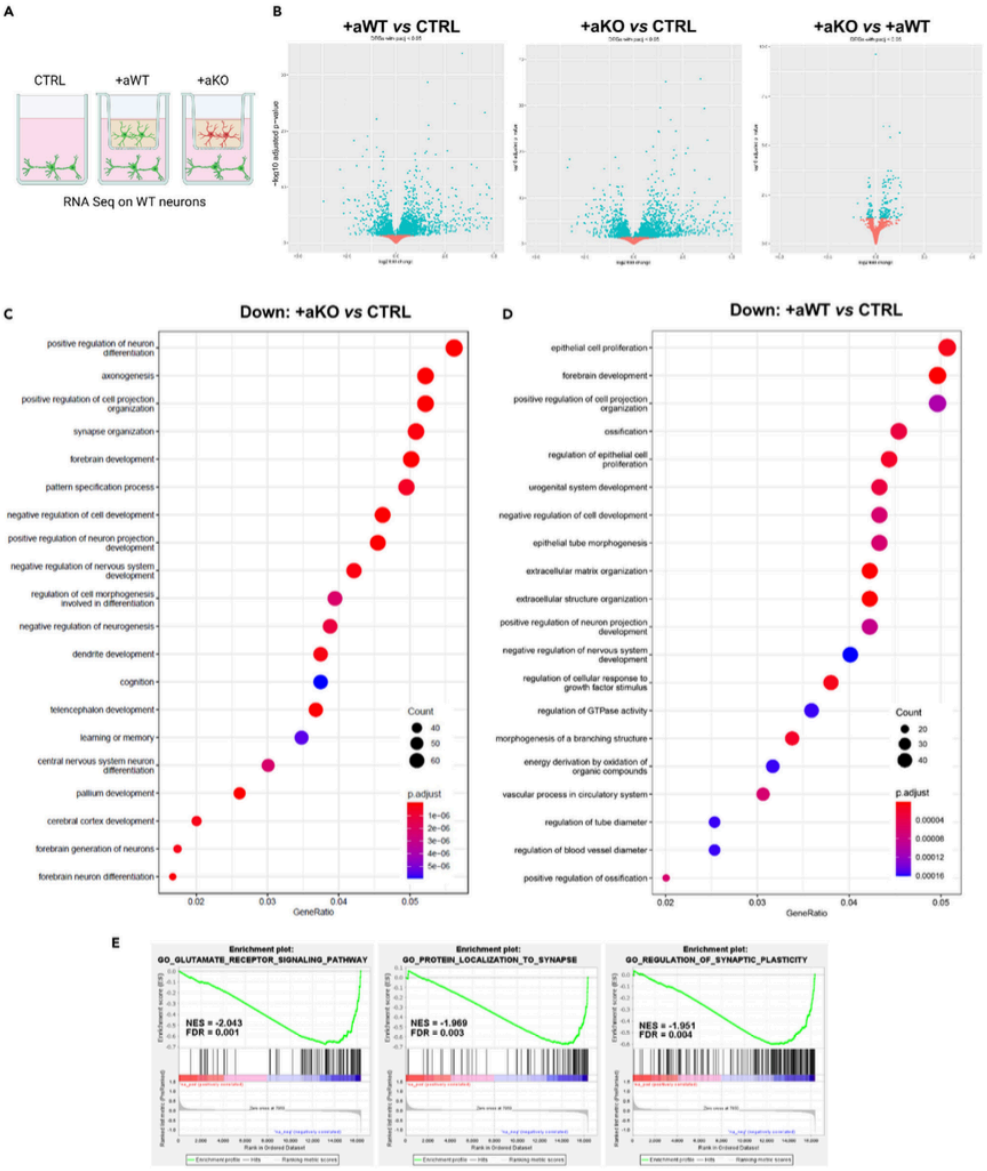

Figure 2. *Mecp2* KO astrocytes affect the expression of genes involved in synaptic maturation in WT co-cultured neurons

(A) Experimental groups included in RNA-seq analysis: neurons cultured alone (CTRL) (n = 5) and neurons matured in co-culture with WT astrocytes (+aWT) (n = 7) or *Mecp2* KO astrocytes (+aKO) (n = 8). Replicate samples were derived from 2 independent experiments.

Figure 2. *Continued*

(B) Volcano plots of DEGs with p.adj<0.05 from the different comparisons, plotted as log_2FC against -log_{10} adjusted p value. Each dot represents a gene: top-right sector highlights genes that are significantly upregulated, top-left sector genes that are significantly downregulated, with p.adj < 0.05.

(C and D) Enrichment analysis of downregulated DEGs at p.adj < 0.05 in +aKO vs. CTRL (C) and +aWT vs. CTRL (D) comparisons, showing the top 20 significant GO terms (biological processes). Size and color of dots represent number of genes associated with each term and FDR adjusted p value, respectively, according to the scale indicated in the figure.

(E) Enrichment plots from preranked GSEA of the comparison +aKO vs. +aWT. Three of the top ten negatively correlated gene sets are reported, together with their normalized enrichment score (NES) and significance (FDR) (see Table S6 for complete results).

(Figure S4D; Tables S7 and S8). Interestingly, many biological processes associated with cytokines production/signaling and immune response were enriched in both GO analyses. To better extrapolate the entity of inflammatory pathways, we exploited Metascape resources on upregulated DEGs,[28] finding that responses to cytokines and inflammatory stimuli were more represented in the +aKO vs. CTRL with respect to the +aWT vs. CTRL comparison (Figure 3A). Moreover, enrichment analysis of transcriptional regulators using the TRRUST database confirmed a stronger activation of transcriptional factors involved in inflammatory response (such as NFkB1, Jun, Stat3, Stat1, Spi1, Ets1, Wnt1, and Bcl3) in the +aKO vs. CTRL comparison (Figure 3B).

To confirm transcriptomic data, quantitative reverse transcription PCR (qRT-PCR) analysis was performed in WT neurons cultured with KO astrocytes, showing an upregulation of genes associated with inflammatory response, including Stat2, Stat3, Chuk, Socs3, and Ikbke (Figure 3C). Furthermore, western blot analysis indicated that protein expression of the inflammatory trigger p65 subunit of NF-kB was significantly increased in neurons cultured with KO astrocytes (Figure 3D). Conversely, the protein levels of Irak1, another component of the NF-kB pathway, that is consistently upregulated in *Mecp2* KO cerebral cortex,[29] were not significantly modified (Figure 3E).

Overall, these data further confirm a defective role of *Mecp2* KO astrocytes in supporting synaptogenesis and point toward a possible role of inflammatory cues' secretion as a candidate molecular mechanism.

Mecp2 KO astrocytes secrete excessive IL-6 and its synthesis depends on neuronal signals

To confirm the activation of an inflammatory-like phenotype in KO astrocytes co-cultured with WT neurons, we measured mRNA levels of a panel of pro-inflammatory molecules in astrocytes cultured with neurons, along with their concentration in the CCM. qRT-PCR showed a strong upregulation of IL-1β, IL-6, and CXCL12 in KO astrocytes. Conversely, the expression of HMGB1, a trigger of inflammation in many neurodegenerative diseases, was slightly reduced (Figure 4A). In parallel, protein levels of the secreted cytokines and chemokines were measured by a cytometric bead-based immunoassay platform, finding a 3-fold increase in IL-6 concentration in the CCM derived from co-cultures with KO astrocytes, and a slight but not statistically significant increase of TNFα, CCL2, CCL3, and CCL4 (Figure 4B).

To verify that KO astrocytes are the main source of secreted IL-6, we measured its mRNA levels also in co-cultured WT neurons. Notably, IL-6 transcripts in neuronal cells were too low to be detected; however, by exploiting our RNA-seq data we did not reveal any deregulation of its expression in co-cultured neurons (Table S1). These results attribute excessive IL-6 secretion exclusively to KO astrocytes.

To assess that KO astrocytes secrete functional IL-6, we analyzed phosphorylation levels of the transcription factor Stat3 in neurons treated for 24 h (from DIV13 to DIV14) with the CCM derived from the co-cultures of WT astrocytes with WT neurons or KO astrocytes with WT neurons. Coherently with flow cytometry data, western blot analysis reported a significant increment of Stat3 phosphorylation in neurons treated with the KO CCM (Figure 4C).

Since the presence of serum in culture might predispose astrocytes toward a reactive phenotype, IL-6 levels were assessed in astrocytes directly isolated from a mouse model of RTT. To this aim, by taking advantage of MACS technology, astrocytes were acutely isolated from the cortex of P7 heterozygous (HET) animals and the corresponding WT female littermates at P7 (Figure 4D), and by qRT-PCR we observed a significant increase of IL-6 mRNA levels in HET astrocytes (Figure 4E). Interestingly, Pearson correlation analysis reported that IL-6 transcriptional levels tend to inversely correlate with the expression of wild-type *Mecp2* ($r^2 = 0.4341$, $p = 0.075$), suggesting that its upregulation might be confined to $Mecp2^-$ astrocytes.

We next proceeded studying whether aberrant IL-6 secretion by KO astrocytes occurs in a cell-autonomous manner. For this purpose, we initially tested if KO astrocyte conditioned medium (ACM) negatively affects synapses. In line with previous publications,[16,18,19] neurons treated with WT ACM showed a healthy phenotype in terms of pre- and post-synaptic puncta number, with a trend toward an increase compared with neurons treated with the control medium. On the contrary, KO ACM caused a detrimental effect on both synaptic compartments. In addition, the lack of any effect in neurons treated with heat-inactivated KO ACM (KO ACM*) demonstrated that secreted neurotoxic factors are thermolabile (Figure S6). However, KO astrocytes cultured alone did not express higher levels of inflammatory molecules. Indeed, qRT-PCR revealed that the expression of IL-6, as well as that of IL-1β and CXCL12, was unaffected, whereas mRNA levels of VEGF, IL17R, EFNB1, IL33, and HMGB1 were downregulated in KO astrocytes (Figure 5A). Accordingly, the lack of Stat3 activation in neurons acutely treated with KO ACM excluded the involvement of IL-6 in the ACM-induced synaptic defects (Figure 5B).

To additionally explore whether IL-6 secretion by KO astrocytes is tightly dependent on the presence of neuronal inputs, we measured by ELISA its levels in the culture medium after neuron removal. In detail, astrocytes were maintained in culture with neurons for 14 days and then transwell inserts were transferred into new plates together with their original co-culture medium. IL-6 was quantified in the medium at DIV14 (before neuronal removal), and then 4 and 8 days after removal of neurons. As expected, we confirmed increased IL-6 secretion when neurons and astrocytes were still in co-culture (at DIV14), whereas a progressive decline of IL-6 concentration was observed after neuron removal. In

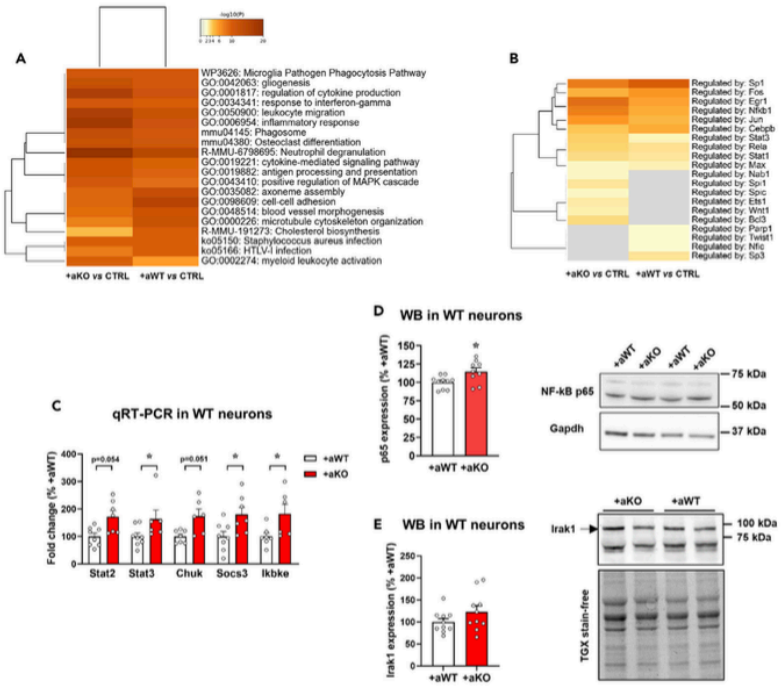

Figure 3. Inflammatory pathways are activated in WT neurons in culture with *Mecp2* KO astrocytes

(A and B) Metascape analyses of upregulated pathways (see Tables S7 and S8) from +aWT vs. CTRL and +aKO vs. CTRL. Top 20 most represented biological processes together with their statistical value (represented as -log10 p value) (A) and enrichment analysis of transcriptional regulators using the TRRUST database (B) are shown.

(C) The histogram reports the mRNA levels of a panel of genes associated with inflammatory responses. WT neurons (DIV14) cultured with KO astrocytes (+aKO, n = 6–7) were compared to WT neurons cultured with WT astrocytes (+aWT, n = 7–8). Data are represented as mean ± SEM and expressed as percentage of +aWT. *p < 0.05, by Mann-Whitney test.

(D and E) Western blot analysis of NF-kB p65 protein levels (D) and Irak1 (E) in WT neurons (at DIV14) cultured with *Mecp2* KO or WT astrocytes (n = 10 +aWT and n = 9–10 +aKO). Data are represented as mean ± SEM and expressed as percentage of +aWT. Representative bands for p65 and Irak1 are reported above the corresponding graph. p65 and Irak1 signals were normalized using Gapdh and total protein content, respectively. Samples derived from 3 independent experiments.

particular, after 8 days, IL-6 levels were significantly decreased and returned to the values measured in the medium of KO astrocytes cultured alone (Figure 5C).

Furthermore, we assessed the influence of neuronal genotype on IL-6 expression, by analyzing its transcription in WT and KO astrocytes cultured with KO neurons. qRT-PCR data reported that *Mecp2* KO astrocytes aberrantly express IL-6 only when sensing WT neurons (Figure 5D), an evidence that strengthens the relevance of studying astrocyte-neuron communication in RTT, that is characterized by a mosaic combination of cells expressing either the WT or mutant *MECP2* allele.

Aberrant IL-6 secretion by *Mecp2* KO astrocytes causes dendritic and synaptic alterations

To evaluate whether the excessive secretion of IL-6 contributes to the observed synaptic defects, an anti-IL6 antibody was added to co-cultures (Figure 6A). We found that inhibiting the IL-6 signaling rescued the number of pre-synaptic terminals, whereas post-synaptic puncta were only partially recovered. Importantly, the same treatment significantly increased the number of colocalized puncta, therefore restoring functional synapses. The specific effect of the blocking antibody against IL-6 was verified by testing an isotypic IgG antibody, which did not produce the same beneficial effects. In line with the physiological role of IL-6 in synaptic formation and maintenance,[30] blocking IL-6 in neurons cultured with WT astrocytes reduced the density of post-synaptic puncta (Figures 6B–6E; DIV14). Of interest, cytokine blockade also rescued the well-known dendritic alterations induced by KO astrocytes in cultured neurons[16,18] (Figures 6F and 6G; DIV6).

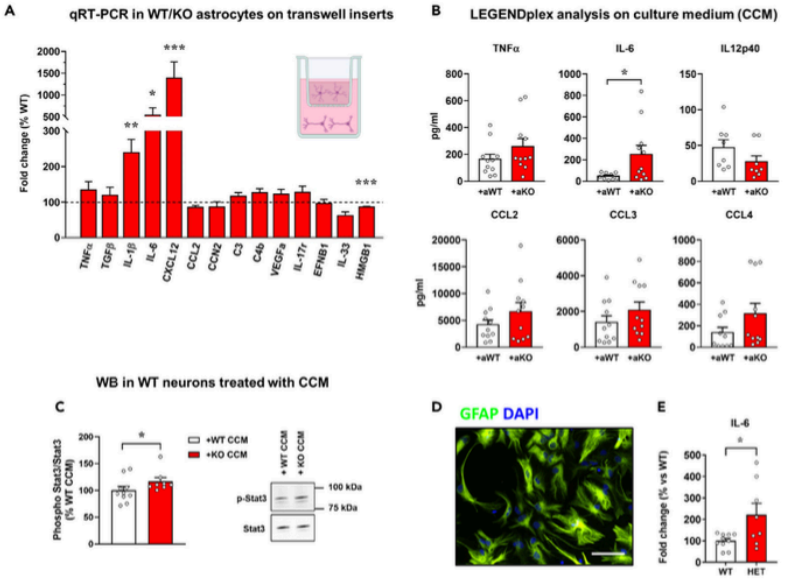

Figure 4. *Mecp2* KO astrocytes secrete excessive IL-6, when in culture with WT neurons

(A) The graph depicts the mRNA expression of selected astrocyte genes in *Mecp2* KO cortical astrocytes cultured for 14 days with WT neurons. Data are expressed as percentages of +aWT condition (n = 10) and represented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 by Student's t test or Mann-Whitney test in accordance with data distribution. Samples derived from at least 3 independent experiments.

(B) Cytokines' quantification in the co-culture medium by LEGENDplex assay kit. Data are represented as mean ± SEM and expressed as percentage of +aWT condition (n = 11). *p < 0.05 by Student's t test. Samples derived from at least 3 independent experiments.

(C) Western blot analysis of phosphorylated Stat3 over Stat3 protein levels in WT neurons treated for 24 h with the medium derived from the co-cultures of neurons with WT (WT CCM) or KO astrocytes (KO CCM). Data are represented as mean ± SEM and expressed as percentage of +aWT (n = 9/10). Representative bands are reported.

(D) Immunofluorescence staining for GFAP in acutely sorted astrocytes from P7 cortices, confirming the purity of astrocyte isolation. Scale bar = 30 μm.

(E) The graph shows the mRNA expression of IL-6 in HET cortical astrocytes, compared to WT astrocytes. Data, expressed as percentage of WT samples, are reported as mean ± SEM. Samples (n = 10 WT and n = 9 HET) derived from 3 independent litters. *p < 0.05 by Student's t test.

Complementary, to evaluate the direct action of IL-6 on neurons, the recombinant cytokine at the dosage deduced by flow cytometry (200 pg/mL) was added to pure WT neuronal cultures every two days, starting from DIV2 (Figure 6H). Immunofluorescence analyses of synaptic puncta indicated that IL-6 led to a significant reduction of the density of pre-synaptic puncta and colocalized puncta, and an almost significant defect in the post-synaptic puncta (Figures 6I–6L).

DISCUSSION

In this study, we investigated astrocyte-neuron communication in the context of RTT by analyzing the ability of *Mecp2* KO astrocytes to regulate synapse formation via paracrine signals. In agreement with literature, our results confirmed the damaging action exerted by *Mecp2* KO astrocytes on neuronal health, corroborating the importance of *Mecp2* expression in astrocytes to sustain neuronal maturation by non-cell autonomous mechanisms.[16,18,31] In addition, by analyzing the density of synaptic puncta, which is profoundly compromised in RTT,[13–15] we demonstrated that *Mecp2* KO cortical astrocytes negatively affect synaptogenesis. Furthermore, we disclosed that molecules secreted by KO astrocytes lead to an abnormal neuronal inflammatory response, triggered by IL-6. Indeed, IL-6 blockade by a neutralizing antibody restores dendritic and synaptic alterations, thus indicating the detrimental action of this cytokine, whose aberrant expression in astrocytes requires a crosstalk with neurons.

Several groups have highlighted the heterogeneity of astrocyte populations and how astrocytes from different brain areas exert distinct effects on neuronal functions.[32] Our results suggest that cortical KO astrocytes may contribute to the altered synaptic density observed in RTT and this evidence is in line with our recent data demonstrating that cortical glial cells show the most severe cytoskeletal and transcriptional alterations in *Mecp2* KO brain.[33]

In the last years, the release of toxic molecules by KO astrocytes has been proposed as a potential mechanism responsible for neuronal defects in RTT and many attempts have been conducted to disentangle such a mechanism with the final goal to develop *ad hoc* therapeutic

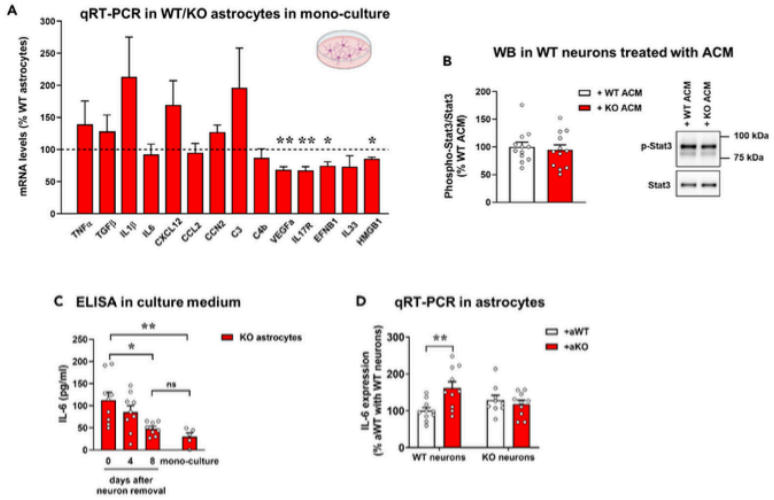

Figure 5. IL-6 secretion by *Mecp2* KO astrocytes depends on neuron-derived inputs

(A) The graph depicts the mRNA levels of selected astrocyte genes in *Mecp2* KO cortical astrocytes cultured alone, expressed as percentages of WT astrocytes (n = 10). Data are represented as mean ± SEM. *p < 0.05, **p < 0.01 by Student's t test or Mann-Whitney test in accordance with data distribution.

(B) Western blot analysis of phosphorylated Stat3 over Stat3 protein levels in neurons exposed to WT or KO ACM. Data, represented as mean ± SEM, are expressed as percentages of neurons treated with WT ACM (n = 12); representative bands for phosphorylated and total Stat3 are reported.

(C) The histograms report the IL-6 concentrations detected by ELISA assay in the medium of KO astrocytes at different days after removal of neurons (0, 4 and 8 days). Data are also compared to those analyzed in the medium of KO astrocytes in mono-culture. Data are represented as mean ± SEM. *p < 0.05, ** p < 0.01 by one-way ANOVA test, followed by Kruskal-Wallis test.

(D) mRNA expression levels of IL-6 in WT and KO astrocytes when in culture with WT or KO neurons are reported as mean ± SEM, and expressed as percentage of WT-WT co-cultures (n = 9–11). **p < 0.01 by two-way ANOVA test, followed by Tukey's post hoc test.

strategies. Diversely from previous studies examining the molecular phenotypes of KO astrocytes alone,[21–23] in order to identify the molecules involved in the occurrence of synaptic defects, we deployed an alternative strategy aimed at assessing deregulated pathways in neurons cultured with astrocytes to deduce upstream regulators. The validity of this approach is supported by the significant downregulation of pathways associated with synaptic maturation and functionality, in line with our immunofluorescence results. Further, the observation that synapse-related pathways were downregulated in neurons cultured with KO astrocytes compared to neurons alone suggested that KO astrocytes release synaptotoxic molecules, although a defective secretion of synaptogenic cues cannot be excluded. Indeed, we believe that a synergic cooperation between reduced secretion of synaptogenic factors and increased release of toxic molecules (among which we identified IL-6) might contribute to the observed synaptic defects.

The novelty of our study also relies on the evidence that the neuronal-astrocyte crosstalk impinges on the *Mecp2* KO astrocyte secretome. Indeed, our molecular data indicated a significant upregulation of IL-6 in KO astrocytes only when maintained in communication with WT neurons, while a decline of IL-6 secretion was observed when neurons were removed. In agreement, previous transcriptomic and proteomic studies performed in KO astrocytes cultured alone never reported an increase of IL-6,[21–23] indicating the importance of astrocyte-neuron communication for dictating the molecular and functional properties of astrocytes. Although mainly neglected in the RTT field, a very recent paper considered the crosstalk between human astrocytes and neurons. The authors reported that the morphological alterations of RTT astrocytes stem from a combination of intrinsic and non-cell autonomous defects, whereas the alterations in gene expression are caused by astrocyte autonomous mechanisms.[19] In contrast, we found that the capacity of neurons to modulate IL-6 expression in *Mecp2* KO astrocytes depends on the genotype of neuronal cells. The observation that increased expression of IL-6 selectively occurs in KO astrocytes when cultured with WT neurons and not with KO ones reinforces the importance of neuronal inputs for the regulation of astrocyte properties.[24] Molecular mechanisms underpinning this fine regulation might rely on neuronal activity and many neuronal-derived signals, including neurotransmitters and neuromodulators,[26] an aspect that deserves further investigations. Intriguingly, these data suggest that molecular differences exist between full KO and heterozygous mosaic tissue, recalling the "cell interference hypothesis" proposed for Pcdh19 mutation, by which the co-existence of Pcdh19-positive and negative cells might affect neuronal interactions.[34]

The upregulation of IL-6 in astrocytes isolated from the cortex of P7 heterozygous animals further strengthens its involvement in RTT and excludes the possibility of methodological artifacts of culturing cells in a medium supplemented with serum. In addition, although in the

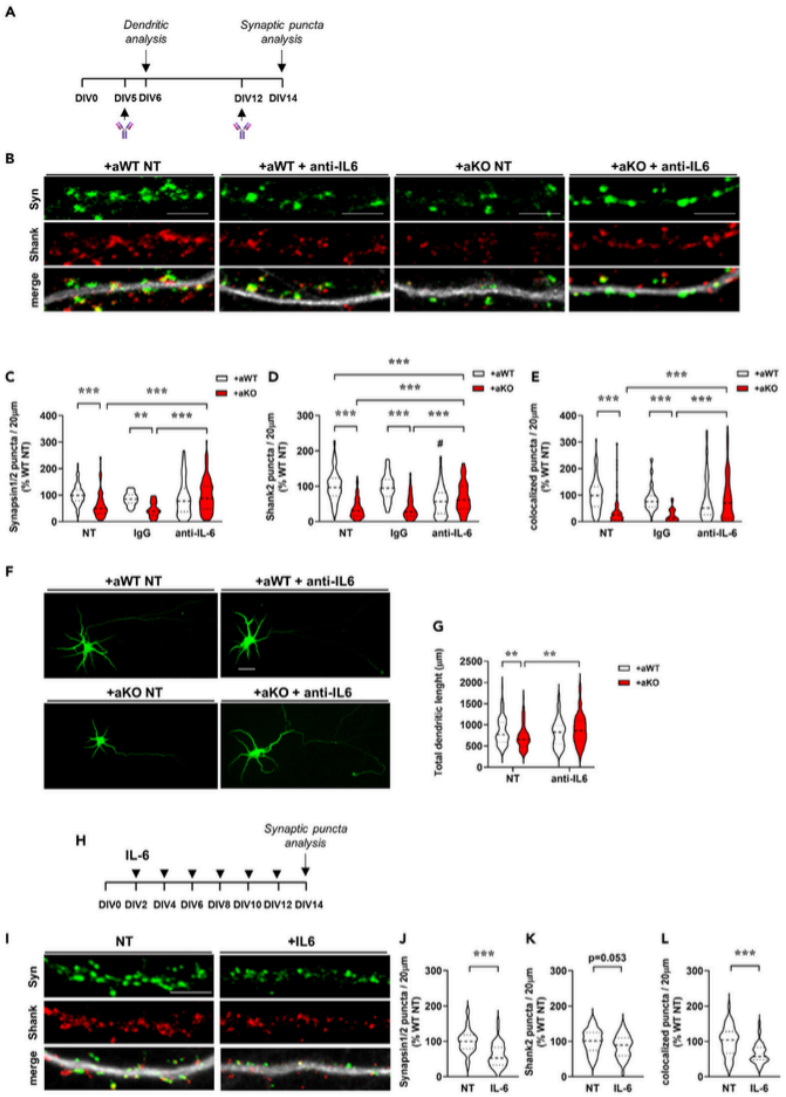

Figure 6. IL-6 released by *Mecp2* KO astrocytes causes dendritic and synaptic defects in WT neurons

(A) Neutralizing anti-IL6 antibody, or an isotypic antibody, was added to co-cultures and analysis of dendrites and synaptic puncta density was conducted at DIV6 and DIV14, respectively.

(B) Representative images of primary branches from WT neurons (DIV14) immunostained for Synapsin1/2 (green), Shank2 (red) and their merge with MAP2 (white). Scale bar = 5 µm.

(C–E) Violin plots indicate the median (dashed line) and 25th and 75th percentiles (dotted lines) of Synapsin1/2 (C), Shank2 (D) and colocalized puncta number (E) in neurons co-cultured with WT (+aWT) or KO (+aKO) astrocytes seeded on transwell inserts. Values for puncta number are expressed as percentages of +aWT untreated condition (NT). **p < 0.01, ***p < 0.001 by two-way ANOVA test followed by Tukey's post hoc test. #p < 0.001 denotes the statistical comparison

Figure 6. *Continued*

between +aWT condition treated with anti-IL-6 antibody versus untreated (NT) or IgG-treated +aWT neurons. Analyses were performed on n > 50 neurons per experimental group from N > 4 biological replicates.

(F) Representative images of Map2 positive neurons (DIV6), when cultured with WT or KO astrocytes and treated with anti-IL6 antibody or left untreated (NT). Scale bar, 20 μm.

(G) Violin plot depicts the total dendritic length in WT neurons cultured with WT (+aWT) or KO (+aKO) astrocytes, following treatment with anti-IL6 antibody or left untreated (NT). Analysis was performed on n > 40 neurons per experimental group from N = 5 replicates. **p < 0.01 by two-way ANOVA test, followed by Tukey's post hoc test.

(H) Recombinant IL-6 (200 pg/mL) was added to WT cortical neurons every 2 days, starting at DIV2. The arrowheads indicate the time of IL-6 treatment.

(I) Representative images of primary branches from WT neurons (DIV14) immunostained for Synapsin1/2 (green), Shank2 (red) and their merge with MAP2 (white). Scale bar, 5 μm.

(J–L) Violin plots indicate the median (dashed line) and 25th and 75th percentiles (dotted lines) of Synapsin1/2 puncta density (J), Shank2 puncta density (K) and puncta colocalization (L) of WT neurons treated with recombinant IL-6. Data are indicated as mean ± SEM. Analyses were performed on n = 40 NT and n = 41 treated neurons from N = 4 biological replicates. ***p < 0.001 by Student's *t* test.

present work we have not discriminated Mecp2^{+} from Mecp2^{-} astrocytes, the trend of an inverse correlation between *IL-6* and *Mecp2* expressions suggests an increased production of the cytokine by astrocytes expressing the mutant allele.

IL-6 is a cytokine involved in the regulation of neuronal and synaptic functions,[30] and, as occurs for this family of proteins, it exhibits pleiotropic actions depending on its concentration, displaying either neurotrophic properties or detrimental activity.[35] Indeed, several data are available regarding the effects of IL-6 on excitatory synapse formation,[30] which might depend on the source of IL-6 and its concentration. For instance, a recent paper has pointed out that a transient IL-6 elevation at early step of neuronal development promotes glutamatergic synaptogenesis.[36] On the other hand, a chronic production of IL-6 by astrocytes at mature stages led to detrimental neuronal effects, including severe synapse loss and degeneration.[37] In our experimental conditions, we proved the synaptotoxic effects of IL-6 by reporting that the neutralizing antibody against IL-6 can significantly improve synaptic alterations. This antibody binds IL-6 with picomolar affinity, thus inhibiting its biological functioning. Complementary, a chronic treatment with IL-6 on WT neurons led to an impaired synaptic phenotype, confirming the synaptotoxic action of IL-6 on developing cortical neurons. Our results support the importance of a fine regulation of IL-6 levels as demonstrated by the evidence that an abnormal upregulation of its release by KO astrocytes alters the process of synaptic formation in neurons. At the same time, even a decreased IL-6 level in WT co-cultures affects the same process suggesting a protective role of this cytokine.

The relevance of our findings is emphasized by previously published data. First, numerous clinical and experimental data point to the presence of a subclinical inflammatory status in RTT, characterized by cytokine dysregulation and aberrant NF-kB pathway.[29,38–41] Of note, IL-6 increased expression has already been described in the brain, saliva, and plasma of patients suffering from RTT,[39,42] as well as in other neuropsychiatric disorders.[43] Notably, IL-6 has been associated with blood brain barrier (BBB) dysfunction[44] and, recently, a defective BBB integrity has been reported in RTT animal models.[45] Additionally, IL-6 overexpression in the mouse brain correlates with neurological abnormalities[37,46] and, conversely, its inhibition improves social behaviors.[47] The relevance of IL-6 and its pathway for mental diseases is highlighted by the observation that they have been already proposed for the development of novel therapies, which however should aim at attenuating IL-6 activity without completely abolishing it, considering the aforementioned physiological roles.[47,48]

In conclusion, our study identified IL-6 as a synaptotoxic molecule triggered by *Mecp2* KO astrocytes, providing an interesting therapeutic target for RTT and other *MECP2*-related disorders.

Limitations of the study

This study is focused on the molecular characterization of the crosstalk between *Mecp2* KO astrocytes and neurons mediated by secreted factors. However, physical interactions between cells, and not only secreted molecules, concur to cell-to-cell communication, particularly during development. Indeed, besides producing many synaptic modulators, astrocytes also express several adhesion proteins, which mediate astrocyte-synapse interactions at tripartite synapses. Thus, future studies are required, in the RTT field, to investigate the molecular mechanisms activated in cultures where astrocytes and neurons mature in contact, with the final aim to detect other molecules, beyond IL-6. Considering the heterogeneity properties of astrocytes, we have to mention that the heterotypic co-cultures that we used allowed to compare the synaptogenic potential of region-specific astrocytes.[27] However, we cannot exclude that this setting could have masked other alterations proper of the respective homotypic culture. Importantly, an investigation of IL-6 secretion by human-derived RTT astrocytes might reinforce the clinical relevance of our results.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
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  - Materials availability
  - Data and code availability

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2024.109296.

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AUTHOR CONTRIBUTIONS

E.A., M.B., E.F., C.C., F.P., R.G., and A.F. conducted the experiments and data analysis. E.A. and A.F. designed the experiments, prepared the figures, and wrote the manuscript. D.P. provided his expertise in IL-6 mediated signaling, supporting analysis of IL-6 signaling, electrophysiological recordings, and experiments with anti-IL-6 antibody. E.B. provided technical support for MACS Technology. C.B. supported the bioinformatics analyses. C.D.P. contributed to molecularly validate data obtained from transcriptomic analyses. C.D.Q. performed RNA-seq and bioinformatics analyses. N.L. revised the manuscript and assisted in planning, interpreting, and discussing results.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Dissemination of the results

Obtained results have been extensively shared with the scientific community. The work carried out during this research has culminated in the publication of the following a peer-reviewed paper in iScience journal (Cell Press) (237):

- **Mecp2 knock-out astrocytes affect synaptogenesis by interleukin 6 dependent mechanisms.** Elena Albizzati, Martina Breccia, Elena Florio, Cecilia Cabasino, Francesca Maddalena Postogna, Riccardo Grassi, Enrica Boda, Cristina Battaglia, Clara De Palma, Concetta De Quattro, Davide Pozzi, Nicoletta Landsberger, Angelisa Frasca – iScience - 2024 Mar;27(3):109296. https://doi.org/10.1016/j.isci.2024.109296

In addition to journal publication, the results of this research have been presented at various national and international conferences. These presentations provided opportunities to engage with peers, gather feedback, and exchange ideas that further shaped the research.

Below, a summary of my research content both in English and Italian for the general public:

- Rett syndrome (RTT) is a rare and severe neurological disorder that primarily affects girls and is a leading cause of intellectual disability. Over 95% of RTT cases are caused by mutations in the *MECP2* gene. Affected girls grow fine up to 6 to 18 months of life when they start to lose all the acquired motor and cognitive skills. This disorder mainly impacts brain, causing dysfunctions in neurons and the cells that support them, called astrocytes. Normally, astrocytes help neurons grow and communicate, but in RTT they become dysfunctional, failing to properly sustain neurons. This study investigated how astrocytes carrying *Mecp2* mutations affect neurons. One key discovery was that these *Mecp2* mutant astrocytes produce too much of a molecule called Interleukin-6 (IL-6), causing inflammation and damage to synapses (the connections between neurons). Blocking IL-6 improved some synaptic issues, but was not able to fully eliminate the pathological condition of neurons. The study also revealed that astrocytes in RTT produce less cholesterol, which is critical for

synapse formation. Interestingly, adding cholesterol helped to correct synaptic deficits. This research highlights IL-6 overproduction and cholesterol dysregulation as possible therapeutic targets for the development of new therapies for RTT.

- La sindrome di Rett (RTT) è un raro e grave disturbo neurologico che colpisce principalmente le bambine ed è una delle principali cause di disabilità intellettiva al mondo. Oltre il 95% dei casi di RTT è causato da mutazioni nel gene *MECP2*. Le bambine affette da questa patologia crescono normalmente fino ai 6-18 mesi di vita, quando iniziano a perdere tutte le abilità motorie e cognitive acquisite. Questo disturbo colpisce principalmente il cervello, causando disfunzioni nei neuroni e nelle cellule che li supportano, chiamate astrociti. Normalmente, gli astrociti aiutano i neuroni a crescere e comunicare, ma nella RTT diventano disfunzionali, non riuscendo a sostenere adeguatamente i neuroni. Questo studio ha indagato come gli astrociti con mutazioni nel gene *Mecp2* influenzino i neuroni. Una scoperta chiave è stata che questi astrociti producono una quantità eccessiva di una molecola chiamata Interleuchina-6 (IL-6), causando infiammazione e danni alle sinapsi (le connessioni tra i neuroni). Bloccando IL-6 si è osservato un miglioramento di alcuni problemi sinaptici, ma non è stato possibile eliminare completamente la condizione patologica dei neuroni. Lo studio ha inoltre rivelato che gli astrociti nella RTT producono meno colesterolo, che è fondamentale per la formazione delle sinapsi. È interessante notare che l'aggiunta di colesterolo ha contribuito a correggere i deficit sinaptici. Nel complesso, questa ricerca mette in evidenza la sovrapproduzione di IL-6 e la disfunzione del metabolismo del colesterolo come possibili bersagli terapeutici per lo sviluppo di nuove terapie per la RTT.