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Effects of ATM kinase inhibition on brain development

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ABSTRACT

The maintenance of a proper balance between excitatory and inhibitory neurotransmission (E/I ratio) is crucial for correct brain development, function and plasticity. Through its inhibitory action, GABA (gamma-aminobutyric acid) neurotransmitter is the principal regulator of E/I ratio, exerting a precise modulation of excitatory transmission. Conversely, at the early stages of neuronal maturation, GABA operates as an excitatory neurotransmitter directly evoking action potentials. Then, during development, it acquires its typical role of brake for neuronal activity through the fundamental process called "excitatory-to-inhibitory switch of GABA", the postnatal transition of GABA transmission from excitatory to inhibitory, directly related to the action of the potassium-chloride co-transporter KCC2. Defects in GABA switch have been largely described in neurodevelopmental disorders such as epilepsy, autism and schizophrenia. In this context, we have recently unveiled a new role of ATM (Ataxia Telangiectasia Mutated), a protein kinase involved in DNA double strand breaks (DSB) repair, in orchestrating the maturation of GABAergic inhibition. Here we demonstrate that the exposure of wild-type neurons in a "critical window" during development to an inhibitor of ATM kinase activity (KU), a drug already exploited as therapeutic tool in oncology, accelerates the excitatory-to-inhibitory switch of GABA. We show that the molecular mechanism underlying KU effect involves the transcription factor Egr4 and the epigenetic regulator MeCP2, which independently and in parallel boost KCC2 expression both *in vitro* and *in vivo*. The resultant neuronal network exhibits a potentiated inhibitory synaptic transmission and appears resistant to a hyper-excitability paradigm. Surprisingly, we found an increased expression of ATM associated to low levels of KCC2 in *Mecp2*$^{y/-}$ mice, the genetic model of Rett syndrome (RTT), a neurodevelopmental disorder associated to mental retardation. Coherently, KU treatment in *Mecp2*$^{y/-}$ neurons, potentiating Egr4 activity on *Kcc2b* promoter and restoring proper KCC2 expression, rescues the delayed GABA switch and counteracts the pharmacologically-induced hyper-excitability, suggesting that increased ATM levels contribute to the generation of the altered neuronal phenotype in RTT. The results collected in this thesis provide new evidence and molecular mechanisms of ATM crucial role in the physiological development of central neurons and highlight ATM inhibition as a prospective therapeutic tool in neurodevelopmental disorders.

RIASSUNTO

Il mantenimento di un corretto equilibrio tra la trasmissione neuronale eccitatoria e inibitoria risulta cruciale per il corretto sviluppo, funzione e plasticità del cervello. Attraverso la sua azione inibitoria, il neurotrasmettitore GABA è il principale regolatore dell'equilibrio tra eccitazione e inibizione, esercitando una precisa modulazione della trasmissione eccitatoria. Al contrario, nelle prime fasi dello sviluppo neuronale, il GABA agisce come neurotrasmettitore eccitatorio, in grado di evocare potenziali d'azione. Durante lo sviluppo, acquisisce il suo tipico ruolo di freno per l'attività neuronale attraverso il fondamentale processo chiamato "GABA switch", la transizione postnatale della trasmissione GABAergica da eccitatoria a inibitoria, direttamente correlata all'azione del co-trasportatore di potassio e cloro, KCC2. Difetti nella maturazione dell'inibizione GABAergica sono stati ampiamente descritti nei disordini dello sviluppo, tra cui epilessia, autismo e schizofrenia. In questo contesto, abbiamo recentemente scoperto un nuovo ruolo di ATM (Ataxia Telangiectasia Mutated), una proteina chinasi coinvolta nella riparazione del danno al DNA, nella regolazione dello switch del GABA. In questa tesi dimostriamo che il trattamento farmacologico di neuroni wild-type con un inibitore dell'attività chinasica di ATM (KU), un farmaco già utilizzato nella ricerca pre-clinica oncologica, accelera lo sviluppo dell'inibizione GABAergica (i.e. GABA switch). Inoltre, mostriamo che il meccanismo molecolare alla base dell'effetto di KU coinvolge il fattore di trascrizione Egr4 e il regolatore epigenetico MeCP2, che indipendentemente e in parallelo promuovono l'espressione di KCC2 sia *in vitro* che *in vivo*. La rete neuronale risultante mostra un'aumentata trasmissione sinaptica inibitoria e appare resistente a un paradigma di ipereccitabilità indotta farmacologicamente. Sorprendentemente, dimostriamo inoltre che nel topo *Mecp2*$^{y/-}$, modello animale della sindrome di Rett (RTT), un disordine dello sviluppo neurologico associato a ritardo mentale, vi è una maggiore espressione della proteina ATM associata a una riduzione dei livelli di KCC2. Coerentemente, il trattamento con l'inibitore di ATM (KU) in neuroni ottenuti da embrioni *Mecp2*$^{y/-}$ normalizza il ritardato sviluppo GABAergico e contrasta l'ipereccitabilità indotta farmacologicamente, aumentando l'attività di Egr4 sul promotore del *Kcc2* e ripristinando quindi la corretta espressione di tale proteina. Questi dati suggeriscono che l'aumento di espressione di ATM contribuisce alla generazione delle disfunzioni neuronali tipiche della RTT. I risultati raccolti in questa tesi forniscono

nuove evidenze e chiariscono i meccanismi molecolari riguardanti il ruolo fondamentale di ATM nello sviluppo del sistema nervoso centrale (SNC) e mettono in luce l'inibizione dell’attività chinasica di ATM come un nuovo potenziale strumento terapeutico nei disordini dello sviluppo neurologico.

INTRODUCTION

Chapter 1

The excitatory/inhibitory balance: implication for neurodevelopmental disorders

The correct brain building during early development requires the appropriate interplay between intrinsic gene expression, molecular pathways and neuronal activity [1-4], with a good amount of external and environmental factors [5]. It is well accepted that the development of the proper brain functions depends on the maintenance of a series of physiological balances including i) cell growth/cell differentiation, ii) neurotrophins production, iii) oxidant/anti-oxidant equilibrium, iv) neurotransmitters release and v) pro/anti-inflammatory molecules [6-9]. Among these, the production and release of neurotransmitters responsible for the precise excitatory/inhibitory transmission (E/I balance), is crucial for a proper circuitry formation, cortical layer organization, activity-dependent tuning of neuronal network and mechanisms at the basis of neuronal plasticity [10-12]. In fact, dysregulation of the E/I balance is proposed to underlie cognitive and social deficits typical of neurodevelopmental disorders (NDDs) [13, 14]. Coherently, most of NDDs such as Autism Spectrum Disorder (ASD), Intellectual Disabilities (ID), schizophrenia, Down syndrome, Fragile X and Rett syndrome have been associated to altered neurotransmitters release, aberrant circuitry formation, impaired generation of inhibition and GABAergic pathway alterations at different developmental stages [15-17].

Maturation of GABAergic inhibition and its key role in brain development

In the brain, neuronal circuits consist of two classes of neurons: excitatory projection neurons, mainly using glutamate as neurotransmitter, and inhibitory interneurons using gamma-amino butyric acid (GABA) as neurotransmitter [18]. Traditionally, GABAergic interneurons have been considered as simple regulator of neuronal excitability, which by their inhibitory action operate as a brake for excitatory neurotransmission [19]. However,

in the last few years, the GABAergic system is getting increasing attention since among the crucial role in modulating E/I balance, inhibitory neurons play a vital role in the regulation of all the key developmental steps in brain maturation, from neuronal proliferation, migration and differentiation to experience-dependent organization and plasticity of local circuits [20-25]. Not surprisingly, alterations in GABAergic development have been related to many neurodevelopmental and neuropsychiatric disorders including schizophrenia, autism, Tourette's syndrome, epilepsy, Down syndrome, Fragile X and Rett syndrome [26-29].

The comprehension of GABAergic interneurons function and maturation is challenged by their heterogeneity. In fact, there are different subtypes of interneurons that exhibit distinct morphology, connectivity pattern and physiological properties [30]. The canonical classification divides interneurons into three largely independent sub-populations based on the expression of calcium-binding proteins: parvalbumin (PV), somatostatin/calbindin (SST/CB) and calretinin/vasoactive intestinal peptide (CR/VIP) expressing neurons. In addition, diverse subtypes of interneurons innervate distinct domains of the post-synaptic glutamatergic cells: PV interneurons specifically innervate the soma and proximal dendrites, whereas SST/CB interneurons selectively innervate distal dendrites of target neurons. This precise spatial organization of inhibitory synapses along principal neurons, highlights specific functional roles carried out by diverse interneuron sub-populations [31].

The formation of the GABAergic system can be divided into distinct developmental steps that include the generation of specific GABAergic subtypes, the migration of these precursors in the appropriate brain region, the formation of synaptic contacts towards specific post-synaptic targets and the activity-dependent adjustment of GABAergic synapse number and strength [18] (*Figure 1*). While the first stages are strongly regulated by genetic

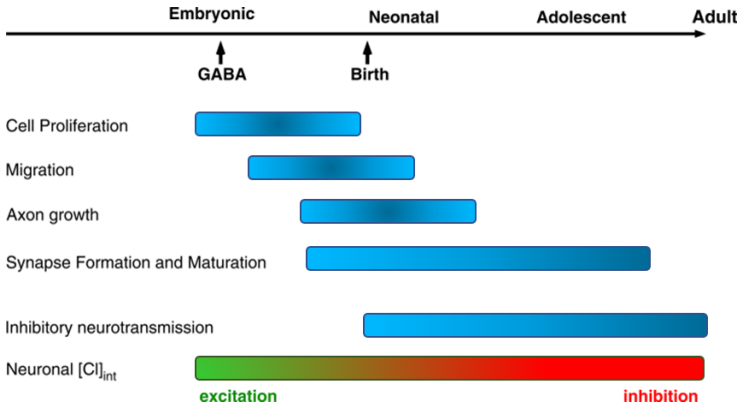


Figure 1. *Time course of GABAergic circuit development in rodents. In the immature brain, GABA-releasing synapses are formed before glutamatergic contacts and represent the first form of communication between neurons. Modified from Di Cristo, 2007.*

programs, synapse maturation is deeply orchestrated by experience [32]. Indeed, the rich pattern of innervation typical of GABAergic neurons is not completed until late adolescence, both in rodent and primates. During this temporal window experience-dependent cellular and molecular mechanisms finely sculpt GABAergic network architecture and control maturation and plasticity of inhibitory synapses [33, 34]. The first molecule shown to be involved in this process is the Brain-derived neurotrophic factor (BDNF), which promotes the formation of GABAergic synapses and adjust their strength and number in both cortex and hippocampus [35].

More recently, it has been strongly demonstrated that GABA signaling itself can promote GABAergic maturation and innervation [36, 37]. This trophic action of GABA on cell proliferation, migration and differentiation lasts from the embryonic period up to perinatal period, or even adolescence, and is principally due to its depolarizing action [38]. In fact, at early stages of neuronal development, GABA acts as an excitatory neurotransmitter, able to evoke action potentials, trigger calcium influx and signaling [39-41]. Then, during brain maturation, we assist to a change of GABA response polarity from excitatory to inhibitory in a fundamental process called "excitatory-to-inhibitory switch of GABA". This change of the electrophysiological and biochemical properties of GABAergic transmission has been shown first in the hippocampus and then also in neocortex and hypothalamus [42, 43]. The switch of GABA polarity is primarily determined by the electrochemical gradient of Cl^-, which is tightly controlled by the action of Na^+-K^+-$2Cl^-$ (NKCC1) and K^+-Cl^- (KCC2) co-transporters [44] (*Figure 2*). In the immature brain, NKCC1 is highly expressed and determines high Cl^- intracellular concentration, so when GABA binds $GABA_A$ receptor, Cl^- flows out promoting

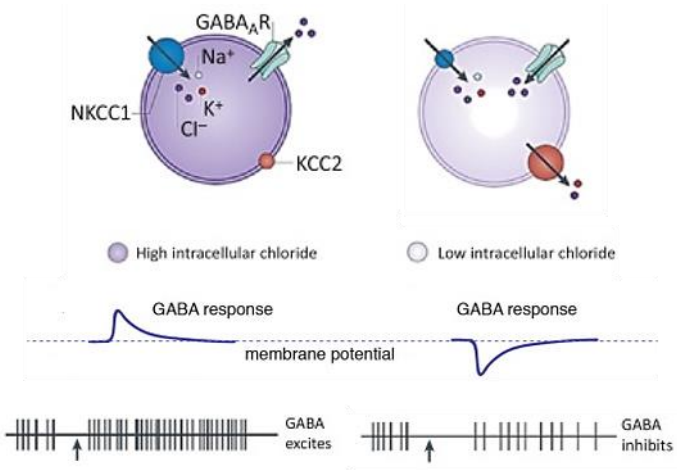


Figure 2. *Schematic diagram of the developmental switch of GABAergic transmission from excitatory to inhibitory. In immature neurons NKCC1 expression is high while KCC2 is low, resulting in high $[Cl^-]_i$ and a depolarizing effect of GABA transmission. In mature neurons, the situation is reversed: the expression of NKCC1 is low and that of KCC2 is high, resulting in low $[Cl^-]_i$ and inhibitory action of GABA. Modified from Ben-Ari, 2015.*

depolarization. Conversely, in the adult brain, NKCC1 expression is reduced and KCC2 levels increase. The chloride extrusion operated by KCC2 establishes low intracellular Cl^-, with a consequent inhibitory action of GABAergic currents. This developmental chloride gradient inversion has been demonstrated to occur in all neurons of the central nervous system, although its timing can significantly change in different brain regions. In general, evolutionarily older brain structures, such as spinal cord, medulla and thalamus, display a precocious neuronal generation and maturation compared to neocortex and hippocampus [45]. The maturation of Cl^- homeostasis follows the same sequence. In the rat embryo for example, a strong KCC2 mRNA expression is already present at E18 in amygdala, thalamus and hypothalamus. On the contrary, cortex and hippocampus show the earliest KCC2 mRNA expression only postnatally, at post-natal day 15 (P15). In mice the scenario is similar: two days in advance compared to rat embryos [46]. Notably, it has been shown by Tyzio and colleagues that in rodent embryos, shortly before delivery, there is a transient excitatory-to-inhibitory switch of GABA action triggered by the oxytocin released by the mother. They demonstrated that this process is crucial for the proper brain development of offspring since maternal oxytocin inhibits fetal neurons and increases their resistance to insults during delivery [47].

The developmental increase of KCC2 mRNA is tightly regulated by neurotrophic factors among which BDNF, that exerts a positive effect on KCC2 expression [48]. Conversely, mice with mutation in BDNF receptor, the tyrosine receptor kinase B (TrkB) receptor, show reduced levels of KCC2 in the hippocampus [49]. BDNF regulation of KCC2 expression is mediated by the phosphorylation of the extracellular signal-regulated kinase 1/2 (ERK1/2), which determines the activation of the early growth response 4 (Egr4) transcription factor, that in turn causes the activation of the *Kcc2b* promoter [50]. Another transcription factor involved in the regulation of KCC2 expression is the upstream stimulating factor 1 (USF1). The interaction between USF1 and the major E-box binding complex contributes the activation of the *Kcc2b* gene expression in cultured cortical neurons [51]. Coherently, mice deficient in USF1 display spontaneous epileptic seizures, suggesting the crucial role of USF1 in normal brain function [52]. It is clear that alterations in every step that regulates this crucial excitatory-to-inhibitory switch of GABA result deleterious for brain development. GABA switch dysregulation, indeed,

determines a failure of inhibition with a complete disruption of the E/I balance and then strong defects in synapse maturation, function and plasticity.

KCC2 dysregulation in neurodevelopmental disorders: the case of the Rett syndrome

As discussed before, alterations in the pathways that regulate GABAergic maturation strongly affect neuronal function leading to E/I imbalance. In this contest, KCC2 dysregulation is one of the most impacting. For example, it has been shown that KCC2 downregulation increases neuronal activity and contributes to seizures generation [53]. Coherently, loss-of-function of KCC2 has been found in both epileptic patients and in various animal models for epilepsy: KCC2 missense variant KCC2-R952H was identified in an Australian family with childhood onset febrile seizures [54], other mutations such as R1049C, a functional variants coding for human KCC2, might be associated with idiopathic generalized epilepsy [55] and in the lithium-pilocarpine induced status epilepticus mouse model, KCC2 mRNA and protein were found significantly downregulated [56]. In addition, it is largely accepted that an altered KCC2 expression is a common feature of diverse neurodevelopmental and neuropsychiatric disorders such as Fragile X syndrome, Down syndrome, autism, schizophrenia and seems to be a key element in the aetiopathogenesis of these diseases [27, 57, 58]. Among all these, Rett syndrome is the clear example of a neurodevelopmental disorder in which a dysregulation of the E/I balance and KCC2 lower expression lead to the most impacting neurological features of the pathology [59, 60]. Rett syndrome (RTT) is a devastating chromosome X-linked neurodevelopmental disorder. In 90-95% of patients diagnosed, the pathology is caused by loss-of-function mutations in the Methyl-CpG-binding protein 2 (*MECP2*) gene. RTT patients display an apparently normal early development until the age of 6-18 month, but then they undergo to a subsequent regression [61, 62]. The main symptoms usually become clear around 12-18 months and include loss of speech, breathing abnormalities, defects in motor coordination, motor disabilities and cognitive impairment [63]. In addition, epilepsy is present in 80% of RTT patients [64]. MeCP2 is a nuclear protein able to bind methylated DNA (*Figure 3*). It is a multifunctional modulator of gene

expression and its activating or repressing actions depend on the molecular context. Recent evidence however unveils additional roles of MeCP2 as a post-transcriptional regulator of gene expression through microRNA mediated mechanisms. Furthermore, the study of the neuronal defects typical of RTT has offered new insights on the plethora of MeCP2 roles at different developmental stages, MeCP2–DNA interactions and the vital importance of MeCP2 in the regulation of the E/I balance, circuits formation and neuronal plasticity [65].

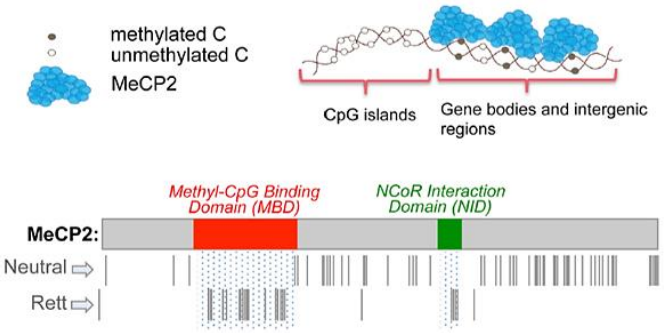


Figure 3. *MeCP2 binds to chromosomes at sites of DNA methylation, present in gene bodies and intergenic regions of the genome. Missense mutations that cause Rett syndrome are concentrated in the MBD and NID of MeCP2. Modified from Cholewa-Waclaw et al, 2016.*

A large amount of evidence clearly points to alterations in the GABAergic system as key pathological features in RTT. Coherently, *Mecp2*-mutant mice, the mouse model of RTT, display a general impairment in GABAergic system [59, 66, 67]. The pivotal role of GABAergic transmission in RTT was directly provided by Chao and colleagues who demonstrated that the selective removal of MeCP2 from GABAergic interneurons, recapitulates most of the pathological phenotype of Rett syndrome [68] and then by Ure et al who rescued RTT disease features only by restoring MeCP2 in inhibitory neurons [69]. Accordingly, MeCP2 is highly expressed in GABAergic neurons and its loss-of-function determines a decrease of GAD65 mRNA and abnormal inter-neuron related genes expression [70]. Importantly, it is largely accepted that the E/I imbalance, typical of RTT, is strongly correlated to a failure in the maturation of the GABAergic inhibition [71]. Indeed, a recent study found low levels of KCC2 in the cerebrospinal fluid (CSF) of Rett syndrome patients [72] and *Mecp2 null* mice display a more depolarized reversal potential of GABA and reduced KCC2 levels, indicating a delay in excitatory-to-inhibitory GABA switch. Accordingly, Insulin-like growth-factor 1 (IGF1) treatment, by enhancing KCC2 expression, rescues behavioural and synaptic deficits in mutant mouse models of RTT [73] and KCC2 overexpression in human neurons derived from RTT patients reverts GABAergic functional defects [60]. The clear link between KCC2 levels and MeCP2 was

further demonstrated by different recent studies which pointed out the direct regulation of KCC2 expression via MeCP2 [60, 74]. In particular, Tang and colleagues showed that MeCP2 prevents the inhibition of KCC2 expression induced by REST. In fact, KCC2 gene has two repressor element-1 sites (RE-1) near its transcription start site and REST, binding to these RE-1 sites, inhibits the expression of KCC2. In wild-type neurons, MeCP2 occupies RE-1 sites in the KCC2 gene, thus preventing REST binding and inhibition of KCC2 expression. Whereas, in Rett neurons, that lack MeCP2, REST binds to the RE-1 sites and suppresses KCC2 expression (*Figure 4*). Because of KCC2 deficiency, Rett neurons show a depolarizing response to GABA.

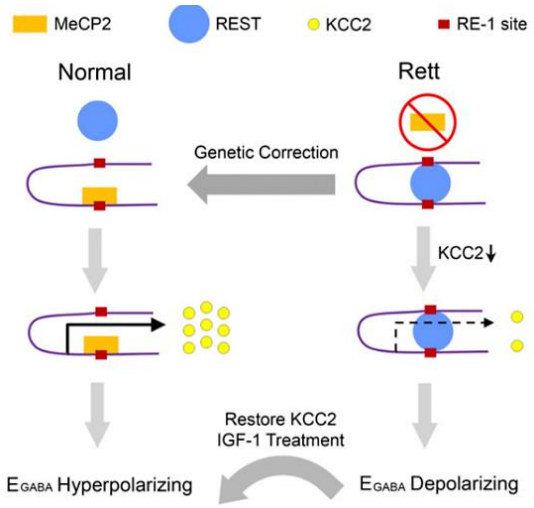


Figure 4. *A working model of the molecular mechanisms underlying KCC2 deficiency in Rett neurons. Modified from Tang et al, 2016.*

All together these studies highlight the importance of the GABAergic system, and especially the crucial role of KCC2 in the aetiopathlogy of Rett syndrome.

Targeting GABAergic system as therapeutic tool in neurodevelopmental disorders

The theoretical model of excitatory/inhibitory imbalance and GABAergic altered development as neurodevelopmental disorders origin, lays the basis for a pharmacological approach based on compounds acting on the GABAergic system at different levels: i) neurotransmitter release, ii) receptor activation and iii) maturation of inhibition [26]. In this scenario, many molecules are already available in clinic such as benzodiazepines and anticonvulsants.

Considering the co-morbidity of neurodevelopmental disorders with epilepsy, anticonvulsants are widely used for the treatments of these pathologies. There are increasing reports showing that anticonvulsant can be useful in ameliorating some autistic-like behaviours [75-77]. Some of these drugs also display an anti-anxiety effect

probably due to the increase of $GABA_A$ neurotransmission by promoting GABA synthesis or inhibiting its re-uptake or catabolism. This is the case of vigabatrin, a GABA transaminase inhibitor, that besides its anticonvulsant action is also able to improve the autistic behaviour of children affected by tuberous sclerosis [78].

Less is published about the efficacy of benzodiazepines in the treatment of ASDs. However, it is widely accepted that gamma-aminobutyric acid agonists are effective in normalizing the E/I imbalance in animal models of ASDs by directly enhancing inhibition or indirectly reducing excitation. As an example, in the animal model of idiopathic autism, i.e. the BTBR mice and in the *Scn1a*$^{+/-}$ genetic model, both characterized by a failure of $GABA_A$ inhibition, the treatment with low doses of benzodiazepines, improve social and cognitive defects only by enhancing GABAergic signaling [79, 80]. Very interestingly, in both animal models is the selective up-regulation of α2 and/or α3 containing $GABA_A$ receptors subunits that improves behavioural defects [79]. Accordingly, new studies are aimed at identifying novel agonists that can selectively target distinct $GABA_A$ receptors subunits to reduce side effects of GABAergic pharmacological treatments.

Although by these pharmacological approaches many phenotypes have been ameliorated in the corresponding animal model, most of studies report only partial rescue of selective impairments with some defects remaining. Given that these disorders are the result of an aberrant development of brain networks, the timing of pharmaceutical treatment could be crucial in the efficiency of correcting impairments. In fact, the focus is now turning to early intervention in NDDs mouse models, in particular on drugs acting on GABAergic development and maturation [29]. In addition, it has been shown that in some children affected by ASDs, benzodiazepines have paradoxical effects, increasing anxiety and aggression [81]. The hypothesis is that GABAergic neurotransmission is still excitatory: enhancing $GABA_A$ mediated transmission does not always means increasing inhibition and could further deteriorate clinical features. Thus, the discovery that, by acting on NKCC1/KCC2 it is possible to indirectly modulate inhibition, opens the way for new therapeutic interventions aimed to correct the defect early during development. That is the case of bumetanide, a FDA-approved loop diuretic NKCC1 inhibitor. In a recent study, Tyzio and colleagues smartly demonstrated that bumetanide application one day pre-delivery to pregnant Fragile X mice or to VPA rats, suppresses excitatory action

of GABA and rescues behavioral features in the offspring in both animal models [47]. Bumetanide has also been tested in human ASD. Following a pivotal study [82], Ben-Ari's group conducted two randomized controlled trials of bumetanide in 60 and 90 children with ASD or Asperger syndrome (3-11 years old). After three months of treatment, children showed significant ameliorations according to the Childhood Autism Rating Scale (CARS), the Clinical Global Impressions and the Autism Diagnostic Observation Schedule [83, 84]. Considering that bumetanide does not pass well the blood-brain barrier (BBB), more lipophilic bumetanide drugs have been developed and tested in animals [85] with promising results also in avoiding the diuretic side-effects due to bumetanide action on NKCC2. On the other side, therapeutic targeting on other components involved in chloride regulation, such as KCC2, is getting increased interest. Recent studies showed IGF-1 as a novel agent able to upregulate KCC2 expression probably via BDNF pathway [86]. Moreover, another recent paper described a new compound, CLP257, as a new tool to counteract hyperexcitability in the central nervous system (CNS). This small molecule can restore chloride concentrations presumably by enhancing KCC2 expression [87]. Thus, the study of mechanism by which chloride concentration is regulated in the brain is getting more attention since the great potential in rescuing pathological phenotypes in neurodevelopmental disorders.

Chapter 2

DNA double-strand breaks (DSBs) and DNA damage response (DDR) in neuronal function and maturation

DNA double-strand breaks (DSBs) are common and unavoidable genetic lesions essential for the survival and evolution of vertebrates. They are generated when both DNA strands are broken at the same position or very close to each other to allow physical dissociation of the double helix into two separate molecules. Despite environmental factors such as ionizing radiation (IR) can induce DSBs, intrinsic factors remain the major source of DNA damage throughout the entire genome [88]. Reactive oxygen species (ROS), continuously formed as by-products of cell respiration, can cause DSBs during all phases of the cell cycle, whereas DSBs are formed only during each S phase in proliferating cells by blocked DNA replication forks or DNA replication through single-strand breaks. Importantly, DSBs induction by tissue-specific nucleases is essential for genetic variability in germ cells during meiosis and it is as much fundamental in the assembly and diversification of antigen receptor genes in developing lymphocytes. Coherently, defects in DSBs repair pathway lead to genomic instability, genetic and epigenetic changes, apoptosis and cell transformation. Thus, cells need to sense and respond to DSBs to survive, maintain cell diversity and function, avoid malignant transformation and guarantee a working immune system [89].

The DNA damage response (DDR) is principally orchestrated by two DNA damage-sensing kinases, ATM (Ataxia Telangiectasia Mutated protein) and ATR (Ataxia Telangiectasia and Rad3-related protein), which are activated by DNA damage and subsequently can phosphorylate hundreds of proteins including the p53 tumor suppressor and other tumor-suppressing and cell cycle-regulating proteins such as CHK2, BRCA1, NBS1 and H2AX [90-93]. ATM itself is a tumor suppressor and is required for vertebrate development; ATR is mainly involved in DNA replication and cell proliferation [94, 95]. The most studied and long-recognized outcomes of DDR signaling are the transient cell cycle arrest coupled with DNA repair, apoptosis, senescence or cancer [96]. Nevertheless, recent studies suggested other potential outcomes especially for developing and mature

neuronal cells. In these cells the DDR appears to yield an unexpected role in controlling cell differentiation programs and even in regulating neuronal activity [97]. This is a diverse function of DDR, completely distinct from the known DSBs response aimed in maintaining genome integrity, suggesting that DDR signaling, and related proteins could exert broader functions in neurons than previously recognized.

Regulation of neuronal differentiation by the DNA damage response

A large amount of evidence clearly suggests a link between neuronal precursor or stem cell (NSC) differentiation and the activation of p53-mediated DDR pathway from DSBs, induced by extrinsic or intrinsic events. In literature is know that IR-induced DSBs determines p53 acetylation in the CNS [98] and the acetylation of Lys320 supports neurite outgrowth *in vitro* and axon regeneration *in vivo* by enhancing Coronin1 and Rab13 signaling [99]. In addition, p53 expression regulation in NSCs located in the subventricular zone (SVZ) is fundamental: loss of p53 results in NCS proliferation, impaired neuronal differentiation and as consequence genesis of glial tumors [100]. Coherently, p53-deficient SVZ NSCs display increased proliferation, defects in maturation and altered expression of neuronal or glial lineage differentiation markers [101]. Importantly, also p21, one of the main targets of p53 signaling, inhibits NSC self-renewal probably by blocking cell cycle progression (*Figure 5*). Thus, p53 deficiency could lessen p21-dependent cell cycle checkpoints causing increasing NSC self-renewal and impaired cell differentiation [102].

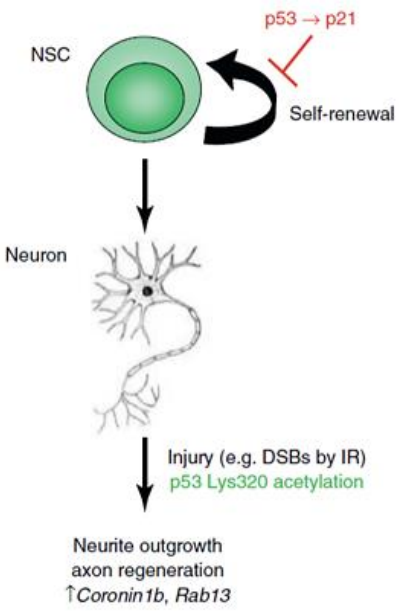


Figure 5. *Schematic representation of p53/p21 signaling downstream of DSBs in neuronal differentiation. Modified from Sherman et al, 2011.*

All together these data highlight a crucial role of p53-dependent DDR in suppressing NSC and multipotent precursor cell self-renewal in favour of differentiation.

DNA breaks and DDR importance in neuronal physiology and disease

Behaviour is strongly influenced by our experience. Neurons are able to sense and process changes fundamental for the survival and for the organism interplay with the external world. This process requires an intricate "dialogue between genes and synapses" in which the exposure to a new stimulus profoundly alters the morphology and connectivity of neural circuits by activating signaling cascades, inducing new protein synthesis and new gene transcription programs [103]. These changes are supposed to underlie the formation of long-lasting memories and adaptive responses since they are crucial for synaptic plasticity. These experience-dependent modifications are governed by the neuronal activity-regulated genes which are classified into diverse subclasses based on the timing of their activation following the activity-dependent stimulus. Genes induced first in this program, the so-called "early response genes", are enriched for transcription factors among which *Fos*, *Npas4*, *Nr4a1*, and *Egr1*. These last, in turn, lead the expression of the so-called "late response genes", such as *Bdnf*, *Homer1*, *Nrn1* and *Cpg15*, which are induced with a longer latency [104]. Together these immediate early gene products regulate experience-driven changes to synapses including synaptogenesis, neurite outgrowth, synaptic strength and maturation and the crucial E/I balance [105]. However, the precise mechanism that coerces early response genes in the absence of a stimulus, and those that trigger their rapid induction, are poorly understood.

Importantly, recent studies pointed out that diverse paradigms of neuronal stimulations, such as the exposure of mice to physiological learning behaviours, determine the formation of DSBs [106, 107]. Madabhushi and colleagues, discovered that these activity-induced DSBs are restricted to few loci through the entire genome enriched for the early response genes *Fos*, *Npas4*, *Egr1*, and *Nr4a1* (*Figure 6*). The mechanism underlying this paradigm seems to depend on a Type

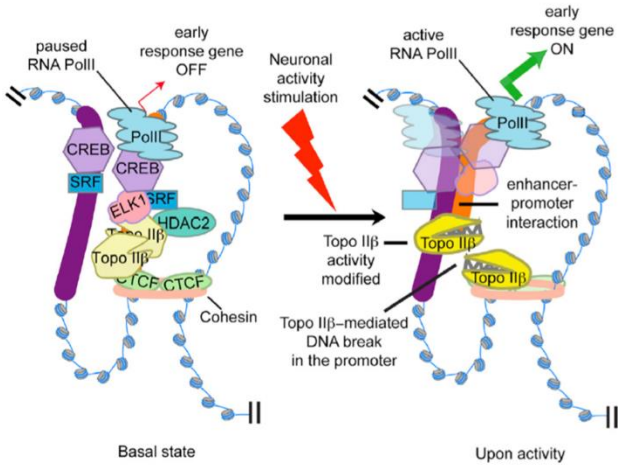


Figure 6. *Neuronal activity triggers the formation of Topo IIβ-mediated DNA breaks in the promoters of a subset of early-response genes that are crucial for experience-driven synaptic changes. Modified from Madabhushi et al, 2015.*

II topoisomerase, Topo IIβ, which is essential for the imminent induction of the early response genes. In fact, *Top2b* null mice display lower activity-induced DSBs and then a decrease induction of early response gene. Thus, engineering targeted DSBs in the promoters of these genes promote their induction even if *Top2b* is knocked down [107]. Together, these results highlight the importance of DSBs formation in the induction of early response genes following neuronal activity and reveal new role of DSBs and related signaling in shaping synaptic plasticity and in controlling neuronal function and behaviour. In addition, it has been shown that Topo IIβ-mediated DSBs induce gene expression also in other cell types and in response to diverse stimulations such as androgens, estrogen and insulin [108, 109]. Thus, this paradigm might be an important and conserved program to rapidly induce gene expression. While early response genes changing in expression are governed by DSBs formation and related repair mechanisms, a recent study showed that *Bdnf* and other late response genes dynamics, are regulated by active demethylation through the base-excision repair pathway [110]. Considering the crucial role of activity-dependent genes expression in neurons, defects in DNA repair have important pathophysiological implications. In facts, loss-of-function mutations in different proteins implicated in DDR generate devasting neurological disorders [111] (*Figure 7*). That is the case of ATR-Seckel syndrome, a complex pathology generated by mutation in *ATR* gene and characterized by microcephaly and progressive aging; LIG4 syndrome, a microcephaly due to defects in p53 dependent pathway and Ataxia Telangiectasia (A-T), caused by mutations of *ATM*, that I will extensively describe in the next chapter.

Human Neurological Diseases Linked to Mutations in DNA Repair Genes

DSBR		
Ataxia Telangiectasia (A-T): ataxia, widespread cerebellar atrophy, occulomotor apraxia, dysarthria, immunodeficiency, and cancer predisposition	*ATM*	*Atm*$^{-/-}$ mice: growth retardation, immunodeficiency, meiotic failure, cancer predisposition, and no obvious cerebellar atrophy. One mutant line shows abnormal motor function (Barlow et al., 1996), the other reveals microglia activation and mild cerebellar degeneration (Kuljis et al., 1997; Xu et al., 1996)
A-T Like Disease (ATLD): ataxia, dysarthria, and occulomotor apraxia	*MRE11*	*Mre11*$^{-/-}$ or *Mre11*$^{H129N/H129N}$ (disrupting nuclease activity) mice: early embryonic lethality, genome instability, although *Mre11*$^{H129N/H129N}$ can activate ATM normally (Buis et al., 2008)
ATR-Seckel Syndrome: microcephaly, dwarfism	*ATR*	*Atr*$^{-/-}$ mice: early embryonic lethality (de Klein et al., 2000); *Atr*$^{S/S}$ (mimicking seckel mutation, a severe hypomorphism): microcephaly, dwarfism, accumulated replicative stress, progressive aging (Murga et al., 2009)
Nijmegen Breakage Syndrome (NBS): microcephaly, immunodeficiency and cancer predisposition	*NBS1*	*Nbs1*$^{-/-}$ mice: early embryonic lethality (Zhu et al., 2001)
LIG4 Syndrome: microcephaly	*LIG4*	*Lig4*$^{-/-}$ mice: late embryonic lethality; p53 dependent apoptosis of postmitotic neuron (Frank et al., 1998, 2000)

Figure 7. *Modified from Madabhushi et al, 2014*

Furthermore, the new role of DSBs in governing the activity-induce gene dynamics suggests that alterations in any step of DSBs, from the formation to the repair, might have a strong impact on cognitive performance. About that, a recent study discovered homozygous mutations in *TDP2* gene in patients with a neurological phenotype characterized by seizures, intellectual disabilities and ataxia [112]. TDP2, the tyrosyl-DNA phosphodiesterase 2, is an enzyme specialized in repairing the 'abortive' TOP2-induced DSBs. Also, DNA repair defects and changes in the expression of protein involved in DDR pathway have been largely descried in neurodegenerative disorders such as Alzheimer disease (AD), Parkinson disease (PD) and Huntington's disease (HD). The hallmark symptom of Alzheimer's disease is cognitive decline; in 1987, Robinson and colleagues demonstrated that alkylation damage is inefficiently repaired in cells obtained from patients with AD [113]. They suggested that this could be the cause of late-onset familial Alzheimer's disease and the associated damage to the CNS. Starting from this pioneering publication, many other studies have shown an increased DNA damage and decreased DNA repair in both patients and mouse models of AD [114]. Recently, Suberbielle and co-workers corroborated this hypothesis showing that transgenic mice for human amyloid precursor protein (hAPP), a well described mouse model of AD, displayed increased neuronal DSBs at baseline and more severe and prolonged activity-induced DSBs after exploration [106]. They demonstrated that i) the suppression of aberrant network activity in hAPP mice normalized their levels of DSBs and ii) Aβ oligomers caused DSBs to form in primary neuronal cultures [106], indicating that transient increases in neuronal DSBs are an integral component of physiological brain activity and that Aβ exacerbates DNA damage by eliciting aberrant synaptic activity. The damage induced by this high amount of DSBs in AD is exacerbated by a defective DDR. In fact, it has been demonstrated a failure of ATM function and consequently a reduced DDR efficiency in both AD mouse model and human AD tissues [115], suggesting that ATM deficiency and altered DDR could be important contributors to the neurodegeneration in AD. Oxidative stress and DNA damage are also implicated in PD, in which the hypersensitivity to DNA-damaging agents seems to be one of the major mechanisms involved in neuronal death [116]. Moreover, HD, a neurodegenerative syndrome caused by mutations of the *IT15* gene encoding for the huntingtin protein, displays a strong impairment in DDR pathway. In fact, HD human skin fibroblast showed

an abnormally low rate of recognized DSBs and irradiated HD cells present a moderate yield of unrepaired DSBs due to a delayed nucleo-shuttling of phosphorylated forms of the ATM kinase probably caused by the binding of ATM to mutated huntingtin in the cytoplasm [117]. Coherently, Lu and colleagues demonstrated an increased ATM signaling in cells derived from HD mice and in brain tissue from HD mice and patients [118]. Also, they showed that the genetic reduction of *Atm* gene dosage by one copy ameliorated multiple behavioural deficits and partially improved neuropathology of HD mice [118], suggesting ATM may be a useful therapeutic target for HD.

All together these findings highlight the enormous importance of studying whether neurological defects arise from defective DSBs and alteration in proteins involved in DNA repair pathways and open the way to new possible pharmacological targets in neurological diseases.

Chapter 3

Ataxia-Telangiectasia

Ataxia-Telangiectasia (A-T) was initially described by Syllaba and Henner in 1926, which documented the first case of A-T in literature. Fifteen years later, the syndrome was again defined by Louis-Bar who described a nine-old boy with progressive cerebellar ataxia and cutaneous telangiectasia [119]. Consequentially, the disease was called Louis-Bar syndrome for a period. The complete description of the pathology was finally provided by Boder and Sedgwick in 1957, which named the syndrome Ataxia-Telangiectasia (A-T), because of the two most visible features of the disease [120]. Only in 1988, using genetic linkage analysis of 31 A-T families, Gatti and colleagues first localized a gene(s) for A-T in chromosomal region 11q22-23 [121]. This finding allows many laboratories to perform extensive positional cloning studies. Thus, the search for A-T gene(s) was reduced to less than 500 kilobases [122]. But not before 1995, one single gene was identified as the cause of the disease [123]. The defective gene in A-T was subsequently named *ATM* (A-T mutated). This gene occupies 160 kb of the entire genomic DNA and encodes a 12 kb transcript of 66 exons [124].

General features of Ataxia-Telangiectasia (A-T)

A-T is a hereditary progressive neurodegenerative rare disorder, with an estimated frequency between 1/40,000 and 1/100,000 live births [125]. It is known that many A-T cases, especially those who die at a young age, are never diagnosed. Therefore, this pathology may be much more prevalent. It is estimated that ~1% of the general population in the United States carries defective A-T genes (according to the A-T Project Foundation). The classical A-T phenotype is caused by homozygosity or compound heterozygosity for *ATM* alleles, which truncate or completely inactivate the protein via missense mutations [126]. Despite the functional uniformity of the mutations, there is a consistent variability in the age of onset and in progression of symptoms. Milder forms

of A-T, which are characterized by later onset and slower progression, are associated with leaky splicing mutations or promoter defects that leave residual amounts of functional ATM protein [127].

A-T generally appears early in childhood: around the age of three patients begin to have trouble in walking independently [128] and by the end of the adolescence become wheelchair bound. Besides the classical A-T signs which are ataxia (lack of muscle control) and telangiectasia (abnormal dilation of capillary vessels), A-T includes a wide range of symptoms, among which: progressive dysarthria (dysphasic disorder of language characterized by difficulty in articulating words), choreoathetosis (abnormal movements of the body), oculomotor apraxia (incapability to follow moving objects), growth retardation, genomic instability that caused an increased cancer susceptibility, hypersensitivity to ionizing radiation and sterility [129-131].

A progressive and massive cortical cerebellar degeneration related mostly to the Purkinje and the granule cells has been described in patients [132] and it is associated to an inexorable loss of cerebellar function. In addition, autopsies and magnetic resonance imaging (MRI) studies have shown significant thinning of the cerebellum and cerebellar atrophy [133]. Finally, degenerative changes in other CNS regions appear during patients' life among which dentate and olivary nuclei, basal ganglia, brain stem and spinal cord. Despite the neurodegeneration, A-T also affects the immune system. In patients an absence or a reduction of various immunoglobulins including IgA and IgE isotypes, IgG2, IgG4 has been described [134]. These alterations are associated with thymic hypoplasia, loss of follicles in the lymph nodes, absence of delayed hypersensitivity reaction and slow formation of circulating antibodies. This state of immune deficiency explains the obvious susceptibility of A-T patients to recurrent lung infections and bronchiectasis [135].

A-T is fatal, affected individuals usually die by their twenties with few surviving into thirties. The cause of death is usually pneumonia or chronic lung disease, which may result from defects in chewing and swallowing caused by the progressive neurological impairment [136]. Neoplasms are frequent, and they are the second leading cause of death [137]. In clinic, diagnosis of A-T is commonly based on the recognition of the classical hallmarks of the pathology: progressive ataxia and telangiectasia. A more precise diagnosis is provided by the analysis of the cellular phenotype of A-T. In fact, cell lines derived from patients show characteristic abnormalities, including defective growth,

increased serum growth factor requirements, premature senescence, major sensitivity to IR and failure to establish effective cell-cycle arrest after genotoxic insult [138]. This method is more accurate, but it requires time and effort for the generation of the cell-line.

No curative strategy for A-T exists and treatments are focused on slowing the progression of the neurodegeneration as well as other approaches are involved in fighting tumors. However, since it seems that oxidative stress may be one of the most important causes of degeneration in A-T, potential therapies based on antioxidant could be promising [139].

Cognitive defects in A-T patients

As previously described, the main neurological defects associated to A-T patients consist in the progressive cerebellar ataxia and in symptoms involving the extrapyramidal pathways such as ocular apraxia and a progressive axonal somatosensory polyneuropathy with an onset peaking around eight years. However, a cognitive phenotype was also described in A-T.

It is widely accepted that cerebellum participates in the organization of higher order functions and cerebellar cognitive affected syndrome (CCAS) is characterized by deficits in executive function, linguistic processing, working memory and spatial cognition, resulting in overall intellectual impairment [140]. The intellectual impairment reported in cerebellar and cerebrocerebellar diseases differs in term of behavioural and cognitive phenotype depending on the pathology [141]. Moreover, the timing and the onset of the condition influence the functional outcome with most important impact in the early stage of development. That is the case of A-T, in which the mutation affects the CNS since the early embryonic stage. Coherently, even pediatric patients display executive function defects, acquire expressive and receptive language impairments, visuospatial disabilities and verbal working memory defects [142]. Despite these defects could be entirely ascribed to cerebellar syndrome, patients also display defects in attention, late learning and memory that cannot be completely attributed to cerebellum. Accordingly, Nora D. Volkow and colleagues [143] have conducted a study to measure brain glucose metabolism by positron emission tomography and 18-fludeoxyglucose in homozygous

and heterozygous patients comparing to healthy controls. By these analyses, they showed that not only homozygotes but also heterozygotes have a reduced glucose metabolism in some brain regions including the hippocampus, the brain structure fundamental for memory and learning functions. This suggests that in homozygous and even in heterozygous asymptomatic patients, mutations of *ATM* gene impact not only cerebellar functions but also properties of different brain regions such as hippocampus.

In this scenario, Mostofsky and colleagues [144] demonstrated a significantly lower verbal IQ and considerable problems with judgment of duration in 17 A-T patients. These neurological defects flank speech abnormalities, difficulty in coordination and timing. Therefore, another team has recently studied a group of eight affected children to understand whether they possess global learning disabilities and/or cognitive impairment or language specific defects and if they were subjected to a progressive course [145]. The results of all of these studies showed that almost all patients have progressive cognitive deficit and that the IQ of younger affected, tested with a non-verbal test, seems to fall into the average (IQ=100) while that of older fits in values between 57 and 83. The analysis of the neuropsychological profile of all the patients, however, showed significant defects of attention, verbal memory, non-verbal memory, late learning and fluency of speech.

Atm$^{y/y}$, a mouse model of A-T

Several model systems have been generated for an appropriate investigation of A-T, but none recapitulate all the features of the pathological phenotype. Most of these lines have been developed in mice. Although *Atm*-deficient mouse models manifest much of the phenotype described in A-T patients, the wide consensus is that they do not exhibit the most debilitating hallmark of A-T, neurodegeneration [146]. Independently developed *Atm*-deficient mouse models may vary in their phenotypic manifestations due to different mutagenesis strategies and/or genetic backgrounds, however they all display immunodeficiency and immune system defects including T-cell maturation, growth retardation, germ cell dysfunction and infertility, cancer predisposition and increased sensitivity to ionizing radiation (IR) [134, 147, 148].

Among all the genetic mouse model generated for A-T, that generated by Borghesani and colleagues, the *Atm*$^{y/y}$, is the closest to A-T pathological phenotype [149]. *Atm*^{y} mutation was generated by replacing the exons 50-52 of human *ATM* gene (the Rad-3 homology domain), with a neo^{r} (neomycin-resistance) gene in murine ES cells (Figure 1). This mutation was transmitted into mice. *Atm*$^{y/y}$ allows to produce two alternative transcripts. In *Atm*^{y} transcript I, the splicing of exon 49 to the exogenous exon results in a frameshift and a stop codon. In *Atm*^{y} transcript II, exon 49 is directly spliced to 53, and the resulting protein presents a working phosphatidylinositol 3-kinase domain. Although these aberrant transcripts can be proved by RT-PCR in *Atm*$^{y/y}$ tissues, neither full-length nor partial ATM-immunoreactive protein bands has been detected [149]. Likewise, in A-T patients with missense or in-frame deletions in which altered ATM protein products may be found [150]. *Atm*$^{y/y}$ mice display pathological phenotypes still present in other *Atm*-deficient mouse models including immunodeficiency, infertility, growth-retardation and they are prone to develop thymic lymphomas. In contrast to others, *Atm*$^{y/y}$ mice also exhibit some aspects of cerebellar pathology reported in A-T patients such as histological changes in the cerebellum as reduced molecular layer thickness, abnormalities in Purkinje cells dendritic arborization and localization. Nevertheless, no evidence for cerebellar degeneration has been detected in this mouse model with age [149]. Accordingly, *Atm*$^{y/y}$ mice exhibit a range of neurological abnormalities and a reduced performance in motor-learning tests. For example, the use of rota-rod testing to measure motor function have shown that wild-type mice have significantly longer latencies to falling-off than mutant animals. They also fail to improve with repeated trials on accelerating, rotating rod. Moreover, spontaneous locomotor activities, measured using the standard open field test, is also significantly reduced in *Atm*$^{y/y}$ mice [146].

All these data demonstrated that *Atm*$^{y/y}$ mouse model is the most suitable for the study of A-T in mice because it recapitulates most and more severe of A-T pathological phenotype.

Chapter 4

ATM (Ataxia Telangiectasia Mutated)

The structure of ATM

ATM is a large Ser/Thr-protein kinases of approximately 350 kDa. It belongs to the superfamily of phosphatidylinositol 3-kinase-related kinases (PIKKs). The PIKK family also includes six other protein kinases among which ATR and DNA-dependent Protein Kinase (DNA-PK), that show sequence homology to phosphatidylinositol 3-kinases (PI3Ks) [135, 151]. ATM contains diverse characteristic domains: an amino-terminal substrate-binding domain (HEAT repeat domain) that binds to some known substrates such as Nijmegen Breakage Syndrome-1 (NBS1), p53 and Breast Cancer Susceptibility Protein-1 (BRCA1), the deletion of this crucial binding domain inactivates the protein [23]; a FAT domain, so named because it is a common motif in other related proteins such as FRAPP/ATM/TRRAP, which interacts with ATM's kinase domain to stabilize the C-terminus region of ATM itself; the kinase domain (KD); the PIKK-regulatory domain (PRD) and an extreme C-terminal FATC domain that represents an ordinary carboxy-terminal amino-acid sequence usually located near the termini of the protein [152, 153]. These last two domains regulate the KD (*Figure 8A*). Although no structure for ATM has been solved, it seems that the overall shape of ATM is very similar to DNA-PKcs and composed by a head and a long arm able to wrap the double-stranded DNA after a conformational change [154] (*Figure 8B*).

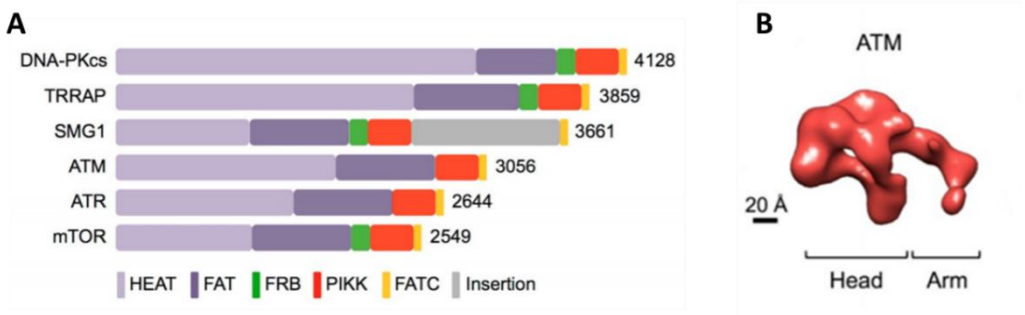


Figure 8. A) *Graphical representation of the domain organization and the total number of residues for each PIKK family member.* ***B)*** *EM (electron microscopy) structure of ATM. Modified from Rivera-Calzada et al, 2015.*

ATM governs genomic integrity and controls cell-cycle check points

Until now, the best-known function of ATM is the DNA repair induced by DSBs. It is at the peak of a signaling cascade that responds to DNA double-stranded breaks (DSBs) and it is required to coordinate the resulting cellular response [155]. ATM is also necessary for processing the physiological DNA strand breaks that happen during meiosis, immune system maturation and for telomere maintenance. In fact, hypersensitivity to ionizing radiation (X-rays and γ-rays), known to break DNA, was reported in A-T patients after radiotherapy for cancer [156] and in A-T cell cultures [157]. ATM exhibits increased kinase activity in response to IR or to the radiomimetic compound neocarzinostatin but not to UV irradiation [91]. UV irradiation induces relatively less significant DNA damage, whereas IR usually produces severe Double-Strand Breaks (DSBs) inside the genome.

ATM undergoes auto-phosphorylation after DNA damage (*Figure 9*). Upon detection of DSBs, the MRE11-RAD50-NBS1 (MRN) complex is assembled at the DNA damage site and then ATM protein is recruited to the lesion site by association with the MRN complex [158, 159]. This association is necessary for proper DNA Damage Repair (DDR) and disruption of the complex may cause profound physiological deficit. The importance of the relationship between ATM and the MRN complex is unveiled by the similarity of two other disorders related to AT: Nijmegen Breakage Syndrome by NBS-1 mutants and A-T Like disorder (ATLD) by MRE11 mutants [160, 161]. These two pathologies are distinct from A-T although they share many close clinical symptoms and cellular hallmarks. Subsequently, MRN complex acts as an

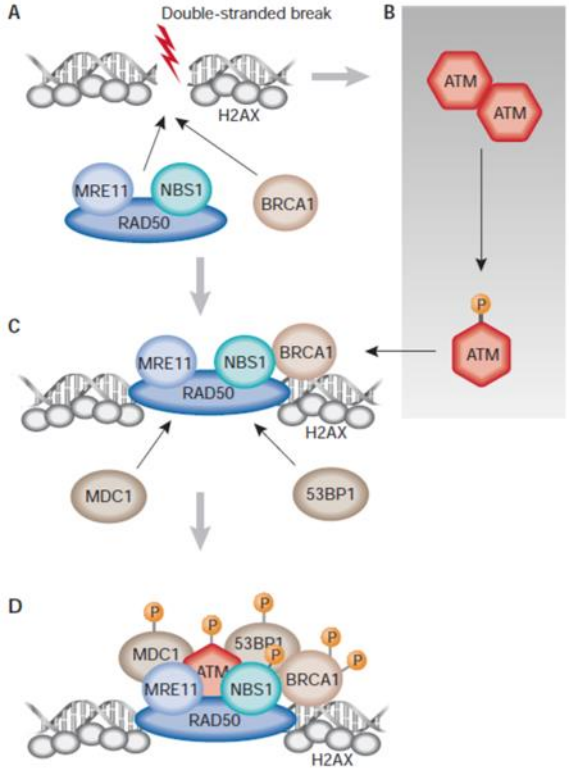


Figure 9. *ATM signalling pathway in response to DNA double-strand breaks. Modified from McKinnon, 2004.*

adaptor for ATM's auto-phosphorylation at Serine 1981 in human (S1987 in mouse). This phosphorylation is fundamental to obtain the dissociation of non-functioning ATM dimer into two active monomers [158, 159]. Once activated, ATM can phosphorylate downstream DDR targets among which the histone variant H2AX, present within chromatin, that is converted into γH2AX by ATM dependent phosphorylation. This seems to be the initial signal for subsequent accumulation of DNA-damage-response proteins including BRAC1 (breast cancer susceptibility protein-1), MDC1 (Mediator of DNA-damage Checkpoint protein) and 53BP1 (p53-Binding protein), which together with the multiprotein complex previously formed, facilitate the cellular response to DSBs [162]. Importantly, MDC1 binds to γH2AX via its BRCA1 C-terminal (BRCT) domain and represents a sort of "master regulator" and initiates the subsequent repair of DSBs [163].

Besides this fundamental role in DNA repair, ATM is also essential in regulating cell-cycle checkpoints (*Figure 10*) [164]. When the DNA double-stranded breaks (DSBs) occur, the inhibition of the cell cycle through the activation of cell-cycle checkpoints is needed for survival [165]. Cell-cycle checkpoints are members of a surveillance system essential to maintain genomic integrity after DNA damage and to avoid the accumulation of mutations due to DNA breaks: if there are any abnormalities, they result in programmed cell death (apoptosis) to eliminate the damaged cell, or they directly arrest the cell cycle to prevent replication of potentially damaged DNA. The cell cycle can stop at different stages: i) before or during S phase, to prevent the DNA replication; ii) before mitosis (in G2 checkpoint) to forestall aberrant segregation of damaged chromosomes. Accordingly, failure of these mechanisms results in genomic instability and cancer predisposition [166]. Because several ATM substrates are key effectors of the cell cycle, cells from A-T patients display defective cell-cycle checkpoints [167-169]. ATM protein is directly involved in cell cycle arrest at both G1 and S phases.

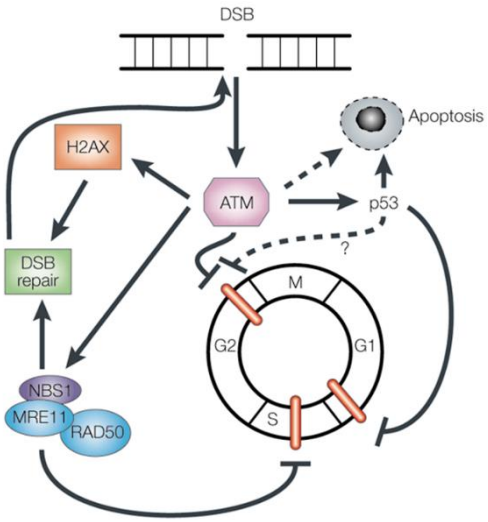


Figure 10. *Scheme of ATM and the cell-cycle checkpoint response to double-stranded breaks. Modified from van Gent et al, 2001.*

The tumor suppressor protein p53 is required for the G1 arrest and its phosphorylation is mediated by ATM on serine 15 after detection of DNA damage. Phosphorylated p53 acts as a transcription-factor that activates the production of p21 (also known as CIP1 or WAF1), which in turn inhibits cyclin E and its partner CDK2 that together form a complex required for progression of the cell cycle from G1 to S phase [170]. Also, Chk2, another target of ATM, is phosphorylated in this process. Once activated, it phosphorylates p53 on serine 20 [171]. ATM also plays a key role in the cell-cycle arrest at S-phase, in which starts DNA replication. In fact, one of the first finding and most important abnormality that characterizes ATM-deficient cells is a failure to arrest DNA synthesis after ionizing radiation. This phenomenon was named Radio-resistant DNA Synthesis (RDS). The S-phase checkpoint arrest depends on a new substrate p95/NBS1. It is a distinct process from G1 checkpoint and does not require p53 [172]. Upon DSBs, activated ATM phosphorylates NBS1 on serine 343. As a result of this NBS1 phosphorylation, the new DNA synthesis site (Replicon) will be inhibited and the cell will be stopped at S-phase [173]. To summarize, upon detection of DSBs, nuclear ATM is activated by auto-phosphorylation and then starts a series of complex programs of cell cycle arrest and/or DNA repair that are necessary to maintain the integrity of the genome.

Chapter 5

Multiple role of ATM protein kinase: beyond the DNA damage response

ATM's involvement in DDR clearly explains many pathological hallmarks of A-T disease including cancer predisposition, hypersensitivity to ionizing radiation, immunodeficiency and infertility [135]. However, neurological symptoms such as ataxia, speech defects and abnormal body movements are still difficult to explain. Indeed, when we regard the CNS phenotype of the ATM-mutant brain, we must conclude that the related pathological mechanism is certainly more complex than a deficit associated only to the DNA repair mechanism [126]. Furthermore, without ATM, cells can still repair the DNA but through processes more time consuming [174]. Anyway, this delay and the enhanced stress related, do not seem enough to explain the neurodegeneration in ATM-deficient humans and in A-T animal models. In accordance, ATM mutations in mice impact on DNA damage response, inducing only mild neurological symptoms [175]. One possible explanation is that ATM may play additional roles in cells of developing and mature CNS in different districts. In line with this hypothesis, many studies revealed the presence of ATM in the cytoplasm, and not only in the nucleus as previously described, playing different, but still important role, then DDR. Thus, while the main presence/function of ATM in the molecular pathways following DNA damage has been explored in depth, many recent studies have demonstrated ATM's involvement in a wider spectrum of cellular activities especially in neurons [176].

ATM as an oxidative-stress sensor

Oxidative stress is defined as a disturbance in the balance between the production of reactive oxygen species (free radicals) and antioxidant defences [177]. Most of the cellular DNA-damages are due to normal metabolic by-product and not to IR or

exogenous radiomimetic chemicals. Indeed, oxidative stress produced by endogenous metabolism causes, at least in part, the constitutive low-level DNA damage response often detected in cultured cell [178]. Because of their intense metabolic activity, neurons are subjected to a considerable oxidative stress, and the deregulation of these processes has been associated with various neurodegenerative conditions [179].

There is consistent evidence that oxidative stress contributes to the A-T phenotype. Accordingly, oxidative stress has been recorded in patients with A-T, in A-T cell cultures and in brain and tissues of *Atm* deficient mice [180, 181]. Moreover, ATM-deficient cells are hypersensitive to oxidative damage and radical scavengers can alleviate this sensitivity [182]. Thus, not surprisingly, neuronal oxidative stress has been suggested as key player in the neurodegeneration process occurring in A-T [183, 184]. All these findings suggest the involvement of ATM in oxidative stress regulation, which goes beyond the "classical role" in DDR. In this scenario, Guo and colleagues smartly revealed that oxidation can directly induce ATM activation in the absence of DSBs [185]. In fact, whereas after DNA breaks, S1981 phosphorylation of ATM leads to ATM activation and dimer dissociation, under oxidation, disulphide bonds are formed resulting in covalent linkages between the subunits of the dimer. In this configuration, kinase activity is found to be activated. Surprisingly this newly discovered activity is independent of S1981 state of phosphorylation and H_2O_2-activated ATM can phosphorylate p53 and CHK2, two traditional ATM targets [185]. Thus, ATM acts as an oxidative sensor and controls redox homeostasis through two main pathways: i) via MAPK pathway and ii) via rerouting of carbon metabolism. Once activated, ATM triggers JNK-cjun pathway [186], which is known to be involved in intracellular redox homeostasis, and regulates central carbon metabolism by promoting the phosphorylation of Hsp27 which in turn activates the Pentose Phosphate pathway and stimulates NADH production [187, 188]. NADH helps in protecting cells against ROS (reactive oxygen species) toxicity because it is a cofactor of many antioxidants enzymes.

This emerging role of cytoplasmic ATM is believed to be crucial in neurons, in which oxidative stress is higher than in another cells types [189]. Thus, cytoplasmic ATM seems to play a neuroprotective activity and the deregulation of this pathway may be one of the main causes of neurodegeneration occurring in A-T patients.

ATM mediates epigenetic regulation

Recent studies have shown another function of ATM probably involved in the determination of the A-T phenotype. By its interaction with HDAC4, a member of Class IIa histone deacetylases (HDACs), ATM seems to act as an epigenetic regulator. It's widely known that class I and class IIa HDACs play important roles in brain development and neuronal survival [190, 191]. HDAC4 is abundant in neurons, especially in Purkinje cells, where it is predominantly cytoplasmic. HDAC4-deficient mice show a significant cerebellar atrophy and Purkinje cells display structural and density abnormalities compared to wild-type. HDAC4 localization depends on its phosphorylation state: the dephosphorylated form accumulates in the nucleus whereas it must be phosphorylated to stay in the cytoplasm [192]. Li J. and colleagues [193], discovered that in samples from A-T patients and *Atm*-null mice, HDAC4 localizes predominantly in the nucleus and this nuclear accumulation is ATM-dependent. In fact, PP2A (Protein Phosphatase 2), the HDAC4 phosphatase, is down-regulated by ATM mediated phosphorylation. Thus, in ATM deficiency, there is an increased activity of PP2A on HDAC4 that cause HDAC4 cytoplasmic deprivation and nuclear accumulation. Consequently, in ATM-deficient neurons global histone acetylation is decreased meaning a closed chromatin configuration and a reduced general transcription. Importantly, HDAC4 directly silences the transcriptional activity of two pro-survival transcription factors: MEF-2 (myocyte enhancer factor 2) and CREB (cAMP response element-binding protein) [194, 195]. The authors also observed a decreased transcription of other crucial neuronal genes such as *Bdnf, NR2a* and *Nrxn*. Thus, the dysregulation of this pathway determines genome-wide alterations in neurons that maybe contribute to neurodegeneration [193].

The same group has recently reported that also histone H3K27 tri-methylation (H3K27me3) mediated by polycomb repressive complex 2 (PRC2), also plays a fundamental role in the development of A-T pathological phenotype [196]. Indeed, EZH2 (enhancer of zeste homolog 2), that is a core catalytic component of PRC2, it has been found to be a new ATM kinase target. ATM-mediated S734 phosphorylation reduces EZH2 stability. Thus, in ATM deficiency, PRC2 formation is elevated along with H3K27me3. This change of H3K27me3 chromatin-binding pattern is strictly related to cell-cycle re-entry and cell death of *Atm*-deficient neurons [196]. Furthermore,

knockdown of EZH2 rescues Purkinje cell degeneration and behavioural alterations in ATM deficient mice, pointing that EZH2 hyperactivity as another key factor in A-T neurodegeneration.

Role of ATM in insulin signaling pathways

Another interesting role of ATM is its involvement in insulin signaling pathways. For the first time, Yang and Kastan demonstrated that ATM kinase activity increases 3-fold in response to insulin in rat 3T3-L1 cells that have been differentiated into adipocytes [197]. Moreover, the phosphorylation of 4E-Binding Protein 1 (an insulin-responsive cytoplasmic protein member of a family of translation repressor proteins) at Ser111 is mediated by ATM and promotes mRNA translation [197]. Thus, cells lacking ATM display a decreased insulin-induced dissociation of 4E-BP1 from eIF-4E with a consequent dysregulation in the insulin-pathway that controls translation initiation.

More recently, it has been demonstrated that ATM is the main activator of PKB/Akt in response to insulin or ϒ-radiations. This effect is mediated by ATM phosphatidylinositol 3-kinase domain that directly phosphorylate Akt at Ser473 (Figure 5), causing the full activation of this transcription factor. Coherently, cell lines derived from A-T patients display altered Akt pathways and *Atm*-null mice show a drastic decrease of Akt phosphorylation in Ser 473 [198, 199]. Moreover, A-T patients are insulin resistant and this could be partially explained by the lack of full Akt activity [200]. Through the response to insulin and other growth factors, Akt plays a key role in many physiological processes such as protein translation, cell proliferation and survival, glucose uptake [201]. Considering its pro-survival function, the dysregulation of Akt pathways plays a pivotal role in cancer development [202] and in this scenario ATM activity on Akt acquires a potential therapeutic effect. It has been demonstrated that blocking ATM activity in cancer cells with overactivated Akt suppresses cell proliferation and induces apoptosis.

ATM implication in synaptic vesicles behaviour in neurons

A very interesting observation about the role of ATM besides DDR regards its association with cytoplasmic vesicles, particularly with synaptic vesicles in neurons. In 1996, Lakin and colleagues found that ATM co-fractionate and co-localize with cytoplasmic vesicles in A-T cell lines [203, 204]. Two years later, another group demonstrated that the cytoplasmic fraction of ATM associates with peroxisomes and endosomes [205] and it is required for the functioning of these organelles. Moreover, the cytoplasmic fraction of ATM does not change in amount or in localization in response to IR [204, 206], meaning that the ATM cytoplasmic pool is distinct from the nuclear one and it is crucial for cellular and sub-cellular activities far from the function in genome surveillance. Importantly, in a pivotal study, Lim and colleagues demonstrated that cytoplasmic ATM binds β-adaptin, a member of the AP-2 adaptor complex, which is involved in clathrin-mediated endocytosis of receptors, membrane trafficking and cell signalling [207]. This interaction was demonstrated *in vitro* but also *in vivo*, by co-immunoprecipitation and co-localization studies. The consequence of the interaction between ATM and β-adaptin is still unclear but indicates that cytoplasmic ATM may play an important role in intracellular vesicle and/or protein transport mechanisms. In fact, several ATM-related lipid kinases such as Vps34 and PI-3K are crucial in stimulating vesicles and protein transport [208]. This also allows to speculate that cytoplasmic ATM may act in vesicle transport and ATM deficiency could deeply impact on protein cellular homeostasis.

In this contest, surprizing discoveries come from studies of cytoplasmic ATM in neurons. In literature, it is known that ATM is predominantly localized in the nucleus of dividing cells, while in post-mitotic cells like Purkinje and granular neurons in cerebellum, it has a significant distribution in the cytoplasm [203, 209]. This hypothesis has been confirmed by Karl Herrup's group [210], which identified cytoplasmic ATM in neuronal cells from cortical structures but not in other peripheral tissue. They also discovered two novel binding partners of cytoplasmic ATM in cortical neurons: VAMP2 and Synapsin-I, known synaptic vesicle proteins that localize in presynaptic nerve terminals. Synapsin-I is a linker protein that keeps synaptic vesicles (SV) related to the cytoskeleton, especially to actin, in presynaptic terminals [211]. Its function is crucial in

maintaining and stabilizing the reserve pool of SVs in the cytoplasm, near the plasma membrane, but also is essential to make SVs ready to be released. It prevents the diffusion and the random fusion of SVs upon repetitive synaptic activity and controls the availability of SVs for the release through its phosphorylation-dependent dissociation from SVs and actin. Thus, its main function is the homeostatic regulation of synaptic transmission [212]. Consistently, synapsin knockout studies have shown that synapsins are not required for neurite outgrowth, synaptogenesis or the basic mechanics of synaptic vesicle release, but they are essential for acceleration of synaptic vesicle trafficking during repetitive stimulation [213, 214]: a failure in synapsin function results in the defective short-term plasticity of synaptic transmission. Vesicle-Associated Membrane Protein2 (VAMP2; also known as Synaptobrevin2) instead, is a central member of the SNARE (soluble N ethylmaleimide-sensitive fusion protein-attachment protein receptor) complex that mediates synaptic vesicle fusion with the cell membrane allowing the neurotransmitter release [215]. The fusion of synaptic vesicles (SVs) with the pre-synaptic plasma membrane is necessary for synaptic transmission at chemical synapses. This fusion event, termed exocytosis, occurs spontaneously at a low and asynchronous rate, whereas an increase in presynaptic Ca^{2+} mediates a larger and synchronous fusion of SVs with the plasma membrane [216, 217]. Crucial to SV exocytosis is the formation of a protein complex between SNARE proteins present on the plasma membrane and SV membrane [218]. In neurons, VAMP2 is a vesicle (or v) SNARE, whereas the plasma membrane proteins SNAP25 and Syntaxin1 act as target membrane (or t) SNAREs. These three SNARE proteins interact to form a trans SNARE complex which is thought to represent the minimal membrane fusion machinery [219]. Thus, VAMP2 is crucial in catalysing SVs fusion reactions and maintaining a proper rate of fusion activities during both spontaneous and evoked synaptic events. The interaction between ATM and these two presynaptic proteins suggests that cytoplasmic ATM might regulate synaptic transmission.

ATM controls E/I balance

In line with the emerging roles of ATM in regulating neuronal functions, in last study published by our group, we unveiled a pivotal role of ATM in controlling the development of the inhibitory system and in maintaining a balanced E/I neuronal transmission at hippocampal level [220]. We demonstrated that hippocampal neurons obtained from *Atm* heterozygous (Het) mouse embryos display an E/I imbalance towards inhibition indicated by: i) a higher frequency of miniature inhibitory post synaptic currents (mIPSCs); ii) an increased number of inhibitory synapses. We revealed that this increased inhibitory tone is due to a precocious development of the GABAergic system, especially to an accelerated "excitatory-to-inhibitory switch of GABA". As I largely described in Chapter 1, the development of GABAergic inhibition is associated to an excitatory to inhibitory shift of the action of GABA because of a reduction of $[Cl^-]_i$, induced by increased KCC2 expression during development [43]. This phenomenon is fundamental for the development of the GABAergic network since it is widely accepted that it regulates the number and the strength of inhibitory synapses. In particular, a precocious GABA switch is known to increase GABAergic synapses and the frequency of GABAergic miniature postsynaptic currents [221, 222]. Thus, in *Atm* Het hippocampal cultures we showed by calcium imaging experiments, an accelerated switch of GABA related to a higher KCC2 expression in hippocampal tissues obtained from *Atm* Het mice. We found that these rearrangements seem to depend to the higher activation of P-ERK and lower expression of its phosphatase, PP1, in *Atm* Het hippocampi [220]. We supposed that the increase of ERK activation could sustain the higher KCC2 expression since in literature is known the link between ERK phosphorylation and KCC2 expression through the Egr4-dependent activation of the *Kcc2b* promoter [50]. These data demonstrate that alterations of ATM expression at hippocampal level determine a precocious and sustained GABAergic development and function leading to an E/I imbalance in favour of inhibition. As I previously described, GABA transmission is strongly related to proper brain development and plasticity, thus this study adds a piece for a clearer comprehension of the molecular basis of brain development introducing ATM as a new key regulator. Moreover, this evidence provides a rationale for the cognitive deficits associated to milder form of A-T,

in which a residual amount of functional ATM is still present and introduces KCC2 and E/I balance as novel potential targets for emerging therapeutic approaches in AT.

In accordance, Herrup and colleagues have recently deepened ATM function at the neuronal synapse. They discovered that ATM and its partner in DDR, ATR, contribute to the E/I balance by regulating synaptic vesicles recycling in cortical neurons [223]. They demonstrated that ATM and ATR specifically segregate to different classes of vesicles playing complementary roles: ATM with excitatory vesicles and ATR with inhibitory ones. They also demonstrated that the two proteins are in balance with each other, so a deficiency of ATM or ATR leads to a compensatory increased expression of the other kinase. This compensatory effect seems to occur rapidly and quickly influences the E/I balance [223].

Chapter 6

Therapeutic relevance of ATM inhibition

Since its central role in DNA damage response, ATM has become an attractive target for cancer therapy. Pre-clinical studies have encouraged the further clinical development of ATM inhibitors, both in combination with chemo- or radiotherapy, to sensitize cancer cells to DNA-damage-inducing agents and to promote apoptosis [224]. In addition, studies showing ATM involvement in insulin pathway, especially in the activation of Akt, have offered new perspectives for using ATM as therapeutic target for cancer treatment. In fact, the activation of Akt pathway is pivotal for cancer development and progression [202].

The emergence of new roles of ATM in controlling neuronal functions [210, 220, 223] has expanded the prospect of using ATM inhibitors also in neurological disorders. Importantly, our recent finding of ATM role in controlling GABAergic development and in the maintenance of E/I balance lays the basis for a "drug-repositioning" of ATM inhibitors as a new tool in the treatment of neurodevelopmental disorders, a topic that I will discuss later in this thesis.

ATM inhibitors: from early non-specific compounds to highly selective small molecules

In parallel to the emerging role of ATM as a potential drug target especially for cancer therapy, in the last 15 years the development of ATM inhibitors has acquired more and more interest, passing from early non-specific compounds to highly selective inhibitors (*Figure 11A-B*). Historically, it has been reported that several methylxanthine-derived drugs (theophylline, pentoxifyllin and caffeine) can sensitize cells to radiations at low millimolar concentrations. The fungal metabolite wortmannin was the first compound proposed to target ATM [225]. It irreversibly inhibits several members of the PIKK family, including mTOR, DNAPKcs and ATM. However, the adverse side effects and

the high dosages (≥10 μM) necessary to obtain radiosensitizating effects, limited its utility. Caffeine is another molecule largely studied and used as radiosensitizing agent although its exact mechanism of action is still unclear [226]. It seems to act inhibiting the phosphotransferase activity of a protein kinase involved in checkpoint signalling of DNA damaged. Nevertheless, the required dose to inhibit ATM activity (IC50 of 0,2 mM) is close to its LD50 *in vivo* and too toxic to permit any use in animals.

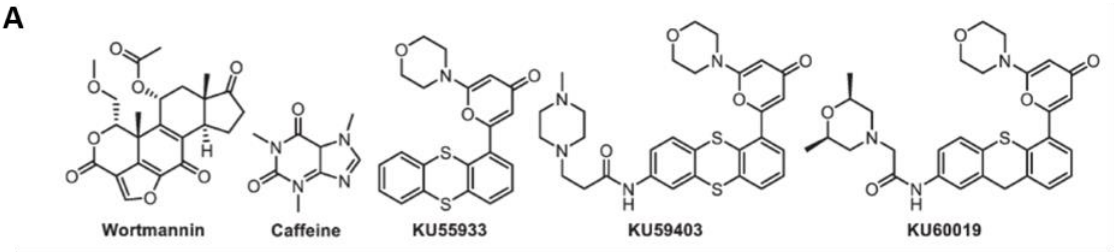
Considering that neither caffeine nor wortmannin are specific and potentially useful *in vivo*, new ATM inhibitors have emerged. High specificity and low dosage has been obtained through small molecules belonging to the "KU family". KU analogues are pyranone compounds that can permeate through cell membranes and act as ATP-competitive inhibitors of ATM with an IC_{50} ranging from 13 nmol/L to 3 nmol/L [227]. These small molecules are extremely selective for ATM, with at least a 100-fold differential in selectivity in a counter-screening against other members of the PIKK family.

Inhibition of ATM kinase activity by KU results in a suppression of ionizing radiation-dependent phosphorylation of several ATM targets, such as p53, ɣH2AX, NBS1, and SMC1 [228, 229]. Consistently, cells exposed to KU display a significant sensitization to ionizing radiation-induced cytotoxicity and to the DNA double-strand break-inducing chemotherapeutic agents including etoposide and doxorubicin [230]. Following this discovery, a large number of studies tested KU effectiveness in radiosensitize different type of tumors such as gliomas [231], non-small cell lung cancer, bladder cancer [232], head and neck cancer [233]. In addition, it has been shown that KU suppresses cell proliferation and apoptosis by abolishing Akt activation in cancer with Akt dysregulated signaling [234].

Nevertheless, KU compounds display a small tissue distribution due to a limited solubility. This represents a big problem especially for brain delivery since the inability to cross the brain-blood barrier [235]. To avoid these hitches several studies have been conducted looking for the better solution to increase KU therapeutic index and solubility *in vivo*. At this purpose, Batey and colleagues identified a novel ATM inhibitor belonging to "KU family", KU-59403, which seems to have good tissue distribution, slow toxicity and significant chemosensitization in *in vivo* models of human cancer [236]. More recently, Wang group developed and evaluated nanoparticle formulations of KU. They

demonstrated that this approach is effective *in vivo*, using a mouse xenograft model of non-small cell lung cancer [237].

A



B

Compound	Enzymatic activity	*In vitro* selectivity	*In vivo* activity	Development stage
Wortmannin	IC_{50} = 150 nM (irreversible)	–	N.D.	Discontinued at optimisation stage
Caffeine	IC_{50} = 0.2 mM	–	N.D.	Discontinued at optimisation stage
KU55933	IC_{50} = 12.9 nM K_i = 2.2 nM	+	N.D.	Discontinued at research stage
KU59403	IC_{50} = 3 nM	++	+	Preclinical stage
KU60019	IC_{50} = 6.3 nM	++	+	Preclinical stage

Figure 11. A) *Chemical structure of ATM inhibitors.* ***B)*** *Table showing principal properties of ATM inhibitors. Modified from Ronco, et al 2017.*

AIM OF THE STUDY

Alterations in the balance between excitatory and inhibitory neurotransmission (E/I balance) have been largely described in neurological disorders such as epilepsy, autism and schizophrenia. E/I balance is mainly controlled by GABA, which through its inhibitory action operates as a brake for excitatory neurotransmission. A fundamental process in the maturation of GABAergic inhibition is the so called "excitatory-to-inhibitory switch of GABA", the postnatal transition of GABAergic transmission from excitatory to inhibitory. In this context we have recently unveiled a new role of ATM (Ataxia Telangiectasia Mutated) in controlling GABAergic development at hippocampal level. ATM is a large protein kinase, whose best-known function is associated to DNA damage response (DDR). Moreover, recent findings have highlighted its important role in neuronal survival and proliferation and in synaptic vesicles recycling. Thus, the absence of the protein may result in neuronal dysfunctions. Coherently, the neurodegenerative condition associated to genetic mutations in *ATM* gene, the Ataxia Telangiectasia (A-T), exhibits, besides cerebellar ataxia, a variable phenotype with cognitive impairment. Consistently, in our previous study, we demonstrated that hippocampal neurons with half amount of ATM protein (*Atm* heterozygous) display a precocious GABAergic development (i.e. excitatory to inhibitory GABA switch) and a higher inhibitory tone, both *in vitro* and *in vivo*, leading to an excitatory to inhibitory imbalance in favour of inhibition.

Thus, starting from these premises, the aim of this thesis was to investigate the possibility to mimic, by a pharmacological intervention, the accelerated GABAergic development and potentiated inhibitory tone typical of the ATM heterozygous phenotype, to propose a new tool for the treatment of neurological disorders associated with developmental alterations and neuronal hyperexcitability. To address this issue, we exploited a potent and selective ATM inhibitor belonging to the "KU family". We performed functional, biochemical and molecular analyses in order to investigate the effects of KU treatment on neuronal maturation and transmission both *in vitro* and *in vivo*. The data presented in this thesis clarify the central role of ATM in proper brain development and highlight ATM inhibition as a new potential pharmacological approach in the treatment of neurodevelopmental disorders.

EXPERIMENTAL PROCEDURES

Animals

All the experimental procedures followed the guidelines established by the Italian Council on Animal Care and were approved by the Italian Government Decree No. 27/2010 and the Italian Legislation (L.D. no 26/2014). All efforts were made to minimize the number of animals used and their sufferings. Wild type mice and transgenic mice for *Atm* and *Mecp2* are under standard conditions of light and temperature, no more than 5 per cage, with food and water ad libitum.

Genotyping

Genotyping for WT/*Atm* Het or KO and WT/*Mecp2*$^{y/-}$ animals was performed on tail extracted DNA, using polymerase chain reaction (PCR) technique. After DNA purification [238], 3 µl of DNA were added to: 7,5 µl of master mix (GoTaq Promega), 0,25 µl of each *Atm* primer 50 µM, or 0,375 µl of each *Mecp2* primer 20 µM and Nulcease free water to reach 12 µl of final volume. The DNA was amplified using thermocycler (Biorad, Hercules, CA, United States).

Primers Sequences for *Atm* genotyping: 5'-TAGGGTGTAGTAGTGGAGGA-3' as reverse primer, 5'-ACGTAAACTCGTCTTCAGACCT-3' as forward primer for *Atm* null allele and 5'-GTAGTAACTATTAGTTTCGTGCA-3' as forward primer for WT allele. Primers Sequences for *Mecp2* genotyping: 5′-CCACCCTCCAGTTTGGTTTA-3′ as reverse primer, 5′-ACCTAGCCTGCCTGTACTTT-3′ as forward primer for *Mecp2* null allele and 5′-GACTGAAGTTACAGATGGTTGTG-3′ as forward primer for WT allele.

Cell Cultures

Characterization of KU impact on neuronal cultures was conducted on hippocampal neurons established from E18 rat littermates as previously described [19]. WT and *Atm* Het neuronal cultures were established from E18 mouse embryos; *Mecp2*$^{y/-}$ genetically-modified mouse neurons were obtained from Postnatal day 0 (P0) pups to minimize the number of females sacrificed. Once obtained, hippocampi were chemically dissociated by treatment with 0.5% trypsin (Invitrogen) for 15 min at 37°C, followed by mechanical dissociation with a fire-polished Pasteur pipette. Dissociated cells were then plated on

poly-L-lysine-treated (1 mg/ml, Sigma-Aldrich) 24 mm glass coverslips in Neurobasal (Gibco) containing 2% B27 (Gibco), 1% Pen-Strep (Invitrogen), 0,5 mM glutamine, 12,5 µM glutamate, as described in Brewer et al. [238], at densities ranging from 10x103 to 20x103 cells/cm2. Neuronal cultures were kept at constant temperature (37°C) in the presence of 5% CO2. At 3 days *in vitro* (DIV), half of the culture medium was replaced with fresh medium without glutamate to avoid excitotoxicity phenomena.

In vitro KU application

The ATM inhibitor KU was dissolved in DMSO at the concentration of 10 mM and diluted in the neuronal medium at the final concentration 1 µM. For chronic treatment KU was applied for four consecutive days, from 8 to 11 days in vitro (DIV), without changing the medium. In the acute protocol neurons were treated at 5-6 DIV for 24 hours and functional properties were measured at 13-14 DIV.

Immunocytochemistry

Primary cortical cultures were fixed with 4% paraformaldehyde and 4% sucrose in 0,12 M phosphate buffer for 20 minutes at 37°C and immunofluorescence staining was carried out as previously described [239]. Images were acquired using a Zeiss LSM 510 META confocal microscope with 60X objective. Immunofluorescence staining was carried out using the following antibodies: guinea pig anti-vGlut1 (1:1000), rabbit anti-vGAT (1:1000), both from Synaptic System, and mouse anti-βIII-tubulin (1:400) from Promega. Secondary antibodies were conjugated with Alexa-488, Alexa-555 or Alexa-633 fluorophores (Invitrogen). The number of vGAT and vGlut1 positive puncta have been counted after the detection of an appropriate threshold which was set to 2.5-fold the level of background fluorescence referring to diffuse fluorescence within dendritic shafts. Fluorescence images processing and analyses were performed with ImageJ Software (National Institutes of Health).

Calcium Imaging

Hippocampal neurons were loaded with membrane-permeable fluorescent Ca^{2+} indicator Fura2-AM (Sigma-Aldrich) 1 µM for 30 minutes at 37 °C, then washed in [Krebs'-

Ringer's-HEPES (KRH) in mM: 125 NaCl, 5 KCl, 1.2 MgSO4, 1.2 KH2PO4, 2 CaCl2, 6 glucose, and 25 HEPES-NaOH (pH 7.4)] and placed into the recording chamber of an inverted microscope (Axiovert 100, Zeiss) equipped with a calcium imaging unit in KRH and tetrodotoxin (TTX, Tocris) 1 µM. Fura2-AM was excited at 380 nm and at 340 nm through a Polychrom V (TILL Photonics GmbH) controlled by the TillVisION software 4.01. Emitted light was acquired at 505 nm at a rate of 1–4 Hz and data collected with a CCD Imago-QE camera (TILL Photonics GmbH). Calcium transients (expressed as F340/380 fluorescence ratio) were calculated in discrete regions of interest (ROIs) of neuronal cell bodies and analysed along sequential images to follow temporal changes.

For "GABA switch" experiments, after a period of baseline acquisition, neurons were stimulated with GABA 100 µM and increments in F340/380 ratio were considered only if higher than 0.05 units. After GABA administration neurons were washed with KRH, let recover few minutes and then excited with KCl 50 mM to identify vital neurons and to analysed Voltage Operated Calcium Channels (VOCC) expression. Transients smaller than 0.1 units were excluded from the analysis.

Chloride Imaging

6 DIV rat hippocampal neurons were loaded with 5 µM MQAE (Biotium) in KRH. MQAE is a 6-methoxyquinolinium derivative and it is used as a fluorescent indicator for intracellular chloride. This dye detects chloride via diffusion-limited collisional quenching. After 1-hour incubation at 37 °C, neurons were washed in KRH and transferred to the recording chamber of an inverted microscope (Olympus) equipped with a CellR imaging station. Image analysis was performed using Xcellence software (Olympus) measuring the mean fluorescence intensity of ROIs.

In vitro electrophysiology

Miniature excitatory and inhibitory post-synaptic currents (mEPSCs and mIPSCs) were recorded in the presence of TTX 1 µM; a reversible blocker of sodium channels that avoids the generation of action potentials. In presence of TTX the mPSCs incur spontaneously, induced by the release of a single vesicle. Thus, they correspond to the event generated from a quantum of neurotransmitter that interacts with postsynaptic

receptors. mEPSCs and mIPSCs were obtained from 13-14 DIV hippocampal neurons in whole cell patch clamp configuration using an Axopatch 200A amplifier (Axon Instruments, Forest City, CA, USA) in the voltage-clamp mode. Currents were sampled at 2 kHz and filtered at 2-5 kHz. External solution [Krebs'-Ringer's-HEPES (KRH)] had the following composition (in mM): 125 NaCl, 5 KCl, 1.2 MgSO4, 1.2 KH2PO4, 2 CaCl2, 6 glucose, and 25 HEPES-NaOH (pH 7.4). Recording pipettes were pulled from capillary glass (World Precision Instruments, Sarasota, FL, USA) using a two-stage puller (Narishige, London, United Kingdom) with tip resistances of 3-5 MΩ, and filled with intracellular solution (in mM): 130 Cs-Gluconate, 10 KCl, 1 EGTA, 10 HEPES, 2 MgCl2, 4 MgATP and 0.3 Tris-GTP. Voltage-clamp recordings were performed at room temperature at holding potentials of -70 mV and +10 mV for mEPSCs and mIPSCs, respectively. Data were analysed off-line (pClamp10 software, Axon Instruments); mEPSCs had to exceed a threshold of -8 pA whereas mIPSCs of 6 pA. The I/E ratio has been calculated by dividing mIPSCs and mEPSCs frequencies measured in the same neuron. MultiUnit activity (MU) has been detected in cell-attached configuration clamping neurons at -60 mV and hyperexcitability was induced applying KRH without Mg^{++} as external solution during the entire recording session. Recording pipettes were filled with intracellular solution (in mM): 130 K-gluconate, 10 KCl, 1 EGTA, 10 HEPES, 2 MgCl2, 4 MgATP and 0.3 Tris-GTP.

Luciferase assay

5 DIV hippocampal neurons were co-transfected with pNL1.1 [Nluc] vector (Promega) containing the -309/+42 region of the KCC2 mouse gene and pGL4.54 [luc2/TK] vector (Promega) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. 2 days after transfection, cultures were briefly washed with PBS and lysed in Passive Lysis Buffer (Promega). Nluc and luc2 luciferase activities were aquired using Nano-Glo Dual-Luciferase Assay System (Promega).

pNL1.1 [Nluc] vector was modified by introducing in its multiple cloning region the -309/+42 region of KCC2 mouse gene containing specifically the Egr4 consensus sequence as unique binding site for transcription factors [50, 240]. Cloning procedures were performed by Bio-Fab Research srl (Rome).

Quantitative real time PCR

6 DIV WT hippocampal neurons were treated with KU 1 µM and lysed prior to RNA extraction with TRIzol reagent (Invitrogen). Total RNA was extracted using the Direct-zol RNA MiniPrep isolation kit (Zymo Research) according to the manufacturer's protocol. The RNA was eluted with 25 µL DNase/RNAse-free water, measured using NANOdrop 2000c spectrophotometer (Thermo Fisher Scientific) and optical density 260/280 nm ratios were calculated. Reverse transcription was performed using 1 µg RNA with a High Capacity cDNA RT kit (Applied Biosystems). Real-time polymerase chain reaction (qRT-PCR) was conducted using a CFX96 thermal cycler (Bio-rad) in a final volume of 10 µL with Sybr Green technique (SensiFAST SYBR Lo-ROX, Bioline). *Mecp2* mRNA was analysed at least in duplicate and data analysis was performed with the ΔΔCt method and expressed as fold change. *Mecp2* mRNA levels were normalized to *Gapdh*.

In vivo KU injection

Post-natal day 4 (P4) C57BL6/J WT pups were anesthetized by cold-ice procedure. After 5 minutes animals received a single unilateral injection of 3 µL of KU 10 µM or DMSO in the lateral ventricle of the right hemisphere. 24 hours later, cortical and hippocampal tissues were explanted from both the ipsi- and contra-lateral hemispheres and stored at -20° C for Western Blotting analysis or fixed of immunofluorescence.

Immunohistochemistry

24 hours after KU injection, pups were euthanized, and brains were explanted and fixed in 4% paraformaldehyde for 48 hours. Tissues were then included in 4% Low Melting Point agarose (Sigma-Aldrich) in 1X PBS. After agarose polymerization, sections of 50 µm thickness were obtained using a VT1000S vibratome (Leica Microsystems). Immunofluorescence staining was carried out on free-floating dorsal hippocampus sections. Staining was performed using primary antibody against Mecp2 (Sigma-Aldrich) followed by incubation with a specific secondary antibody, counterstained with DAPI and mounted with Fluorsave (Merck). Images were acquired in the stratum radiatum of the CA1 subfield of the hippocampus using the x40 oil immersion objective with an

additional electronic zoom factor of up to 3 (voxel sizes of 0.10 × 0.10×1 μm) and maintaining the parameters of acquisition constant in each group.

Western Blotting

Proteins were obtained starting from explanted tissues homogenised in sample buffer (sodium dodecyl sulphate 1% (SDS), 62.5 mM Tris-HCl (pH 6.8), 290 mM sucrose); or from neurons scraped using a lysis buffer containing 3% SDS, 115 mM sucrose, 65mM Tris-HCl (pH 6.8), 0,1 % βmercaptoethanol. Protein content was assessed by bicinchoninic acid (BCA) assay, using BCA protein assay kit (Thermo Fischer Scientific). In this method, BCA molecules chelate cuprous ion Cu^{+}, formed from Cu^{++} reduction by proteins in alkaline environment. This chelation generates a purple-coloured reaction product which has a strong absorbance at 562 nm, proportional to protein concentration in the sample. Sample absorbance was read through a spectrophotometer (Victor2 - 1420 multilabel counter, Wallac) set to 550 nm. Protein content was assessed through a bovine serum albumin-based standard curve. Protein were separated by SDS-PAGE electrophoresis, blotted and incubated with primary antibody followed by HRP-conjugated secondary antibody (Jackson ImmunoResearch) and developed by Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific). The following primary antibodies were used: rabbit anti-KCC2 1:1000 (Millipore), rabbit anti-ATM 1:500 (Millipore), rabbit anti-LC3B1/2 1:1000 (Cell Signaling), HSPA8 1:1000 (Cell Signaling), mouse anti-p-ERK 1:1000 (Sigma-Aldrich), rabbit anti-ERK1/2 1:1000 (Cell Signaling), rabbit anti-Mecp2 1:1000 (Sigma-Aldrich) antibodies. Rabbit anti-calnexin 1:1000 (Sigma-Aldrich), mouse anti-actin 1:1000 (Sigma-Aldrich) or mouse anti- βIII tubulin (Sigma-Aldrich) were used to normalize.

Statistics

Pairwise comparisons between treatments were assessed with Student's t-test. One-way ANOVA with repeated measures or 2-way ANOVA as between subject factor were used. Post hoc analysis was done using Tukey's, Bonferroni's or Holm Sidak's post hoc tests. The differences were considered significant, if $p<0.05$ (indicated by one asterisk), $p<0.01$

(double asterisks), $p<0.005$ (triple asterisks). All statistical analyses were done with software Prism, version 6 (GraphPad).

RESULTS

Characterization of the ATM kinase inhibitor KU in neuronal primary cultures

KU effectiveness and duration of action

In order to investigate how and at which extent the pharmacological blockade of ATM kinase activity could impact hippocampal function, we exploited a potent and selective ATM inhibitor belonging to the "KU family". As I largely described before, KU molecules are small compounds that can permeate through cell membranes and act as ATP-competitive inhibitors of ATM. Until now, these molecules have been largely studied in the oncological field, since it has been demonstrated that ATM inhibition induces cancer cells apoptosis and sensitizes cells to ionizing radiation (IR), improving efficacy of IR therapy in several types of cancer [228-230]. First, we evaluated KU effectiveness and duration of action in hippocampal primary cultures established from rat embryos. To test whether KU is able to penetrate inside neurons efficiently and then inhibits the kinase activity of ATM, 14 DIV (days in vitro) cultured hippocampal neurons were treated for an hour with 20 μM of Etoposide (ETO), to elicit the ATM dependent DNA damage response [241], or 20 μM of Etoposide plus 10 μM of KU. Then, the experimental groups were analysed by Western Blotting experiments to quantify the extent of ATM phosphorylation using the specific antibodies against ATM total protein and P-ATM S1981. While Etoposide treatment clearly activates ATM kinase activity, as showed by the appearance of the phosphorylated form of ATM (P-ATM in red, *Figure 1A*) compared to control (first band, *Figure 1A*), KU 10 μM co-application clearly abolishes this activation (third band, *Figure 1A*). To assess the duration of action of KU in hippocampal neuronal cultures, we carried out Western Blotting analysis. We therefore treated 14 DIV cells with KU 10 μM and we collected samples at different time points: 4 hours, 24 hours or 48 hours after KU application. In all the experimental conditions Etoposide was applied an hour before cell lysis. Western Blotting analyses revealed that KU activity persists up to 24 hours after treatment, then decreases and disappears 48 hours later, when P-ATM/ATM ratio is comparable to Etoposide experimental condition (*Figure 1B*).

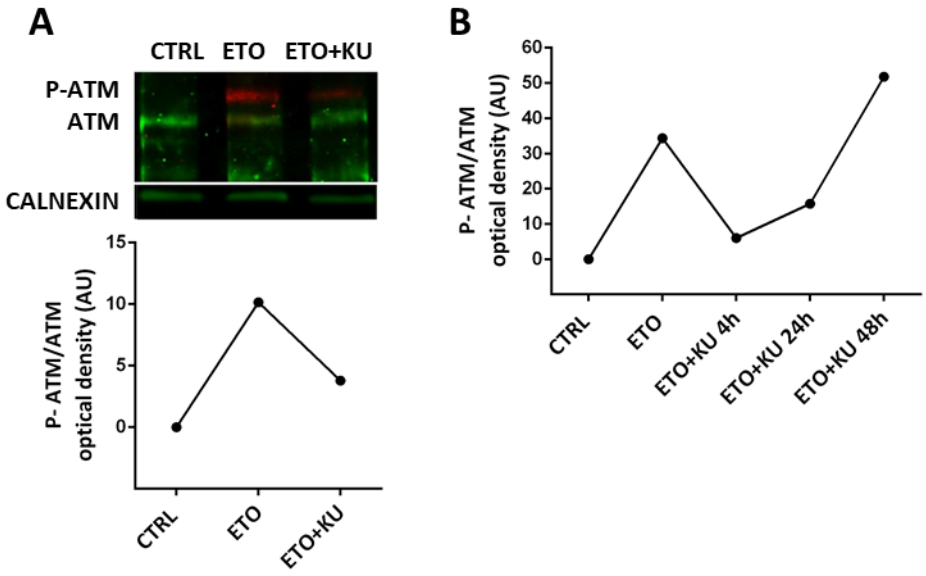


Figure 1. ***(A)*** *Representative image and analysed data of Western Blotting experiments carried-out on 14 DIV rat hippocampal neurons treated with Etoposide (ETO) 20 µM or ETO plus the ATM inhibitor KU 10 µM.* ***(B)*** *Western Blotting analysis of KU duration of action in 14 DIV hippocampal neurons.*

Setting-up KU concentration in neurons

After establishing KU effectiveness and duration of action, we treated neurons with different KU concentrations (1 µM, 5 µM and 10 µM) and both in acute (4 hours) and chronic (4 days) paradigms, in order to individuate the lower dose able to produce the desired effects without affecting neuronal health. Firstly, we performed immunofluorescence experiments on 12 DIV hippocampal primary cultures labelling neurons with βIII-tubulin to better see the morphology and the branching of neuronal processes. As indicated by qualitative immunofluorescence images, the acute treatment (4 hours) with KU 5 µM and 10 µM induces neuronal damage determining the appearance of varicosities along tubulin-labelled neuronal processes (*Figure 2*). The formation of that kind of varicosities has been already described by Borghesani and colleagues, as a first sign of neurodegeneration in ATM-deficient Purkinje cells [149]. Coherently, the chronic treatment (4 days) with KU 5 µM produces neuronal varicosities and deeply affects neuronal survival as indicated by the strong reduction of dendritic arborisations (*Figure*

2). On the other hand, both the acute and the chronic administration of KU 1 µM keeps neuronal processes healthy (*Figure 2*).

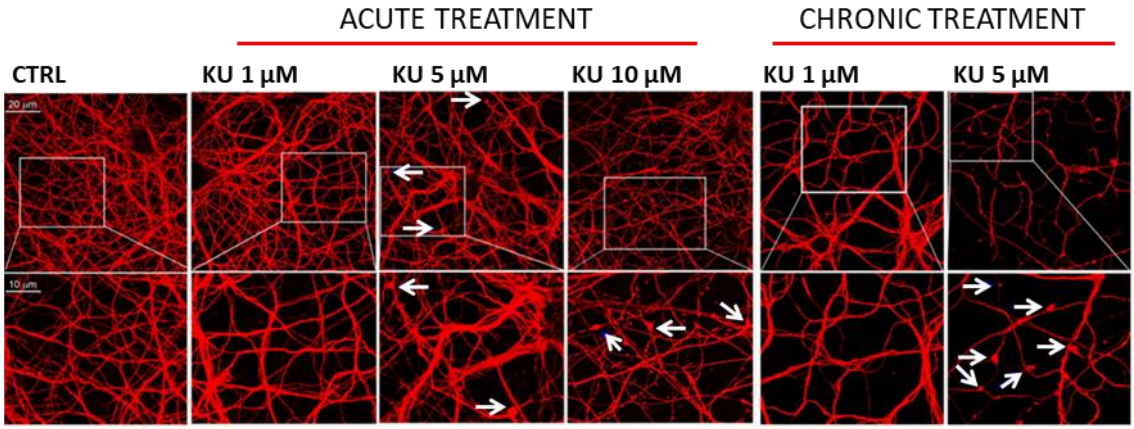


Figure 2. *Representative immunofluorescence images of 12 DIV rat hippocampal neurons labelled with a specific antibody against βIII-tubulin display the occurrence of pathological varicosities along neuronal processes upon both chronic and acute treatments with KU 5 µM and 10 µM. Only KU 1µM - treated cells appear healthy.*

To better investigate the impact of KU chronic treatment on neuronal function and homeostasis, we performed calcium imaging experiments on primary neuronal culture exposed to KU 5 µM or 1 µM for 4 days (from 8 to 12 DIV) and loaded with the ratiometric calcium indicator Fura-2AM. Calcium imaging data revealed alterations in neuronal calcium homeostasis after KU 5 µM chronic treatment, indicated by a higher F340/380 fluorescence ratio, confirming that concentrations higher than 1 µM result in neuronal damage and functional impairments (*Figure 3A*). We also checked for any possible unspecific effect linked to the use of KU 1 µM, but we did not find changes in both intracellular basal calcium levels (*Figure 3A*) and in the expression of Voltage Operated Calcium Channel (VOCC), as indicated by comparable intracellular calcium increases upon KCl stimulation (*Figure 3B*). Moreover, several studies show that KU concentrations even higher than 1 µM display a good selectivity for ATM kinase [242-244]. Thus, we established 1 µM as the ideal KU concentration able to guarantee both safety, selectivity and effectiveness in neuronal primary cultures.

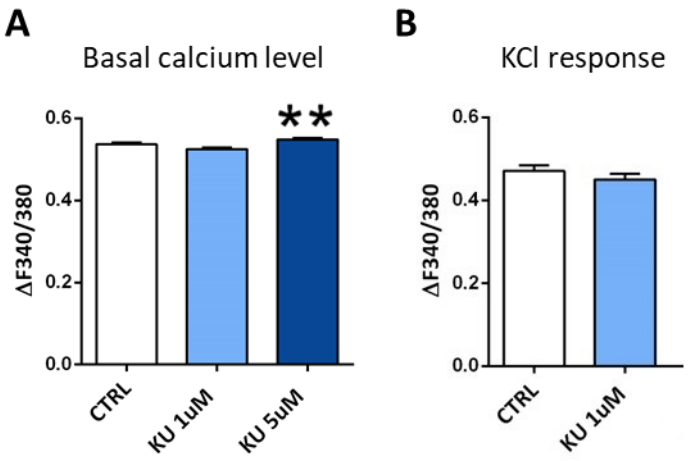


Figure 3. *Calcium imaging experiments conducted on rat hippocampal neurons chronically treated with KU 1 µM or 5 µM from 8 to 12 DIV.* ***A)*** *Quantitative analysis of basal calcium levels. Neurons exposed to the chronic treatment with KU 5 µM display increased resting calcium levels compared to control (Kruskal-Wallis Test followed by Dunn's Multiple Comparison Test, **p<0,01. CTRL n=244; KU 1µM n=221; KU 5µM n=200).* ***B)*** *Calcium transients evoked by KCl 50mM application in neurons chronically treated with KU 1µM vs controls (Mann-Whitney Test, p=0,214. CTRL n=244; KU 1µM n=219).*

Evaluation of functional changes induced by KU treatment in neurons

Neuronal exposure to KU enhances inhibitory neurotransmission and KCC2 expression

Our next goal was to investigate the possible occurrence of changes in basal synaptic transmission after the pharmacological blockade of ATM kinase activity through KU. At this purpose, we daily treated hippocampal neurons from 8 to 11 days in vitro (DIV) with KU 1 µM and we performed electrophysiological recordings of excitatory and inhibitory miniature post-synaptic currents at 12 DIV (*Figure 4A*). Miniature excitatory and inhibitory post-synaptic currents (mEPSCs and mIPSCs) (*Figure 4B*), or MINIs, were recorded in whole-cell voltage clamp in the presence of 1 µM tetrodotoxin TTX (Tocris). TTX is a reversible blocker of sodium channels, that avoids the generation of Action Potentials. In fact, the mPSCs are calcium independent-events which incur spontaneously, induced by the release of a single synaptic vesicle, thus they correspond to the release of "a quantum" of neurotransmitter that interacts with postsynaptic receptors [245]. This analysis allows to specifically measure functional synaptic properties. In particular, the frequency of mPSCs, measured in Hertz (Hz), indicates the number of mPSCs per second and is a parameter strictly related to the pre-synaptic compartment since it is influenced by the number of synaptic vesicles contained in each pre-synapsis, the probability of vesicle release and the number of pre-synaptic compartments that approach the patched neuron. On the other hand, the amplitude of the mPSCs is a measurement of post-synaptic properties, since it directly depends on the number of functional post-synaptic receptors expressed along the membrane [246, 247]. Electrophysiological data showed that the chronic treatment with KU 1 µM potentiates inhibitory synaptic transmission in terms of mIPSCs frequency (*Figure 4C left*) but not of amplitude (*Figure 4E*), indicating a prevalent pre-synaptic effect. Conversely, the excitatory counterpart remains unaffected (*Figure 4C right – Figure 4F*), leading to an excitatory/inhibitory imbalance in favour of inhibition (*Figure 4D*).

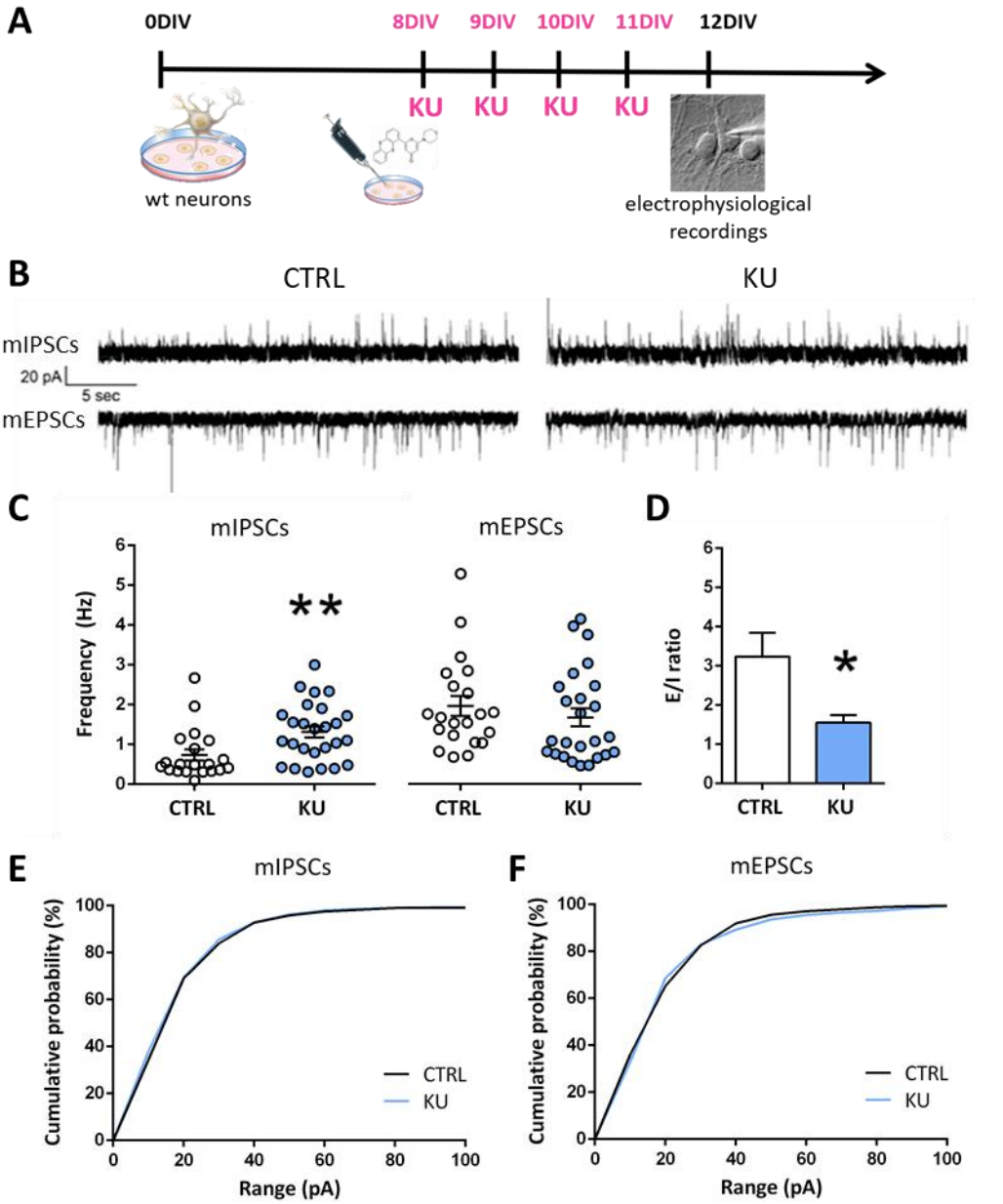


Figure 4. A) *Scheme showing the temporal line of KU chronic treatment in hippocampal cultured neurons.* ***B)*** *Representative electrophysiological traces of inhibitory and excitatory miniature post-synaptic currents (mIPSCs and mEPSCs) measured in 12 DIV neurons chronically treated with KU.* ***C)*** *Electrophysiological analysis of mIPSCs and mEPSCs frequency (mIPSCs: Mann-Whitney Test, **p=0,005; CTRL n=20; KU n=26. mEPSCs: Mann-Whitney Test, p=0,247. CTRL n=21; KU=25).* ***D)*** *Quantification of excitatory/inhibitory ratio (E/I ratio) evaluated by measuring the frequency of excitatory and inhibitory events from each single cell in whole-cell configuration (Mann-Whitney Test, *p=0,027. CTRL n=14; KU n=20).* ***E-F)*** *Analysis of mIPSCs and mEPSCs amplitude plotted as cumulative probability (mIPSCs: KS test, p=0,073. CTRL n (events)=818; KU n (events)=1809. mEPSCs: KS test, p=0,128. CTRL n (events)=1480; KU n (events)=1233).*

Since in our previous study we demonstrated a potentiated inhibitory tone probably linked to an increased KCC2 expression and precocious GABAergic development in *Atm* heterozygous (Het) hippocampal neurons [220], we investigated the hypothesis that the enhanced mIPSCs frequency, induced by the pharmacological blockade of ATM activity through KU, could be ascribed to increased KCC2 levels. To address this issue, we first confirmed the direct link between "ATM kinase activity - KCC2 levels and degree of GABAergic inhibition", taking advantages of the specific KCC2 receptor antagonist, VU0240551 (VU) [248, 249]. The exposure of wild type neurons to VU 1 µM determines a significant intracellular chloride accumulation, measured with the chloride fluorescent indicator MQAE, proving VU effectiveness in blocking KCC2 action (*Figure 5A*). We therefore treated *Atm* Het neurons, which exhibit higher levels of KCC2, with VU 1 µM during development, and we assessed VU rescuing of functional deficits in Het compared to controls. At this purpose, we evaluated the excitatory-to-inhibitory switch of GABA through calcium imaging experiments, as an index of GABAergic development strictly related to KCC2 expression [44], and the E/I balance by electrophysiological recordings (*Figure 5B*). Calcium imaging experiments, performed in 5 DIV neurons loaded with the ratiometric calcium indicator Fura-2AM, revealed that VU treatment is effective in normalizing the anticipated inhibitory action of GABA typical of *Atm* Het cells, as indicated by a comparable percentage of neurons responding to GABA 100 µM stimuli with calcium transients in wild-type (WT) vs Het+VU experimental conditions (*Figure 5C*). In addition, we did not find any changes in calcium homeostasis after VU administration, as showed by the undistinguishable calcium basal levels measured in Het treated cells compared to WT (*Figure 5D*). Only weak alterations in calcium homeostasis have been detected in WT neurons upon VU treatment accordingly to Sivakumaran et al, 2015 [250] (*Figure 5D*). Also, electrophysiological recordings of mEPSCs and mIPSCs from each single cell performed in 14 DIV neurons showed the effectiveness of VU treatment during development in rescuing the increased inhibitory tone, indicated by a normalized mIPSCs frequency (*Figure 5E*), and the imbalanced E/I transmission (*Figure 5F*). All together these data provide the unequivocal proof of the direct link between ATM activity, KCC2 levels and degree of inhibition.

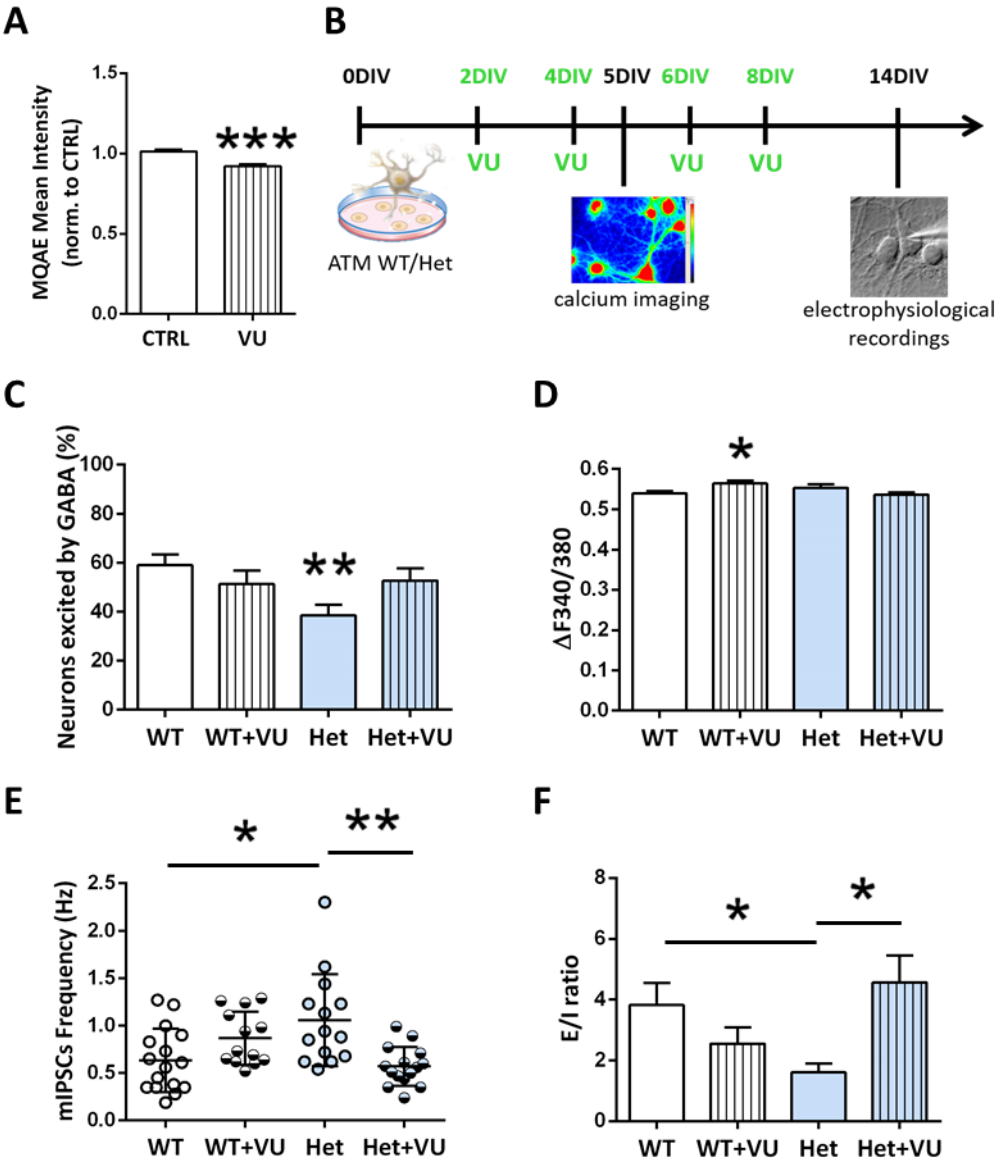


Figure 5. A) *Intracellular chloride measurements in 6 DIV primary hippocampal neurons upon VU0240551 (VU) treatment. Quantitative analysis of the MQAE mean intensity: a lower MQAE mean intensity in VU-treated cultures indicates an increased* $[Cl^-]_I$ *(Student's t-test, ***p<0,001. CTRL n=138; VU n=133).* ***B)*** *Scheme of the experimental time line of VU treatment and related functional experiments.* ***C)*** *Quantitative analysis of the percentage of 5 DIV Atm Het/WT neurons, treated or not with VU, responding to GABA application by depolarization (Kruskal-Wallis Test followed by Dunn's Multiple Comparison Test, **p<0,01. WT n=362; WT+VU=214; Het=342; Het+VU=299).* ***D)*** *Evaluation of resting calcium level in Atm Het/WT cells upon VU treatment (Kruskal-Wallis Test followed by Dunn's Multiple Comparison Test, *p<0,05. WT n=362; WT+VU=214; Het=342; Het+VU=299).* ***E)*** *Electrophysiological analysis of mIPSCs frequency in 14 DIV Atm Het/WT hippocampal neurons treated or not with VU (Kruskal-Wallis Test followed by Dunn's Multiple Comparison Test, *p<0,05 - **p<0,01. WT n=16; WT+VU n=13; Het n=14; Het+VU=15).* ***F)*** *Quantification of E/I ratio in 14 DIV Atm Het/WT hippocampal neurons upon VU chronic treatment (One-way Anova followed by Holm-Sidak's Multiple Comparison Test, *p<0,05. WT n=15; WT+VU n=11; Het n=11; Het+VU=11).*

To investigate if KU effects on GABAergic transmission are due to changes in KCC2 expression, we performed Western Blotting experiments of chronically treated neurons, but we did not observe any statistical significant increase in KCC2 levels (*Figure 6A*). Moreover, we did not find significant changes in KCC2 expression also in 12 DIV neurons treated for 1 hour or 24 hours with KU (*Figure 6A*). So, we hypothesized that we were not in the proper time window to see possible alterations in KCC2 levels since it is well known that at this developmental stage (12 DIV) KCC2 reaches a saturating expression level and GABA switch is almost completed [43, 251]. Thus, we moved earlier during development and we treated cells at 7 DIV, since it is well described that in this phase of neuronal maturation about 40-50% of neurons still respond to GABA with depolarizing calcium transients [181]. We found that at this time point the acute KU application (for 30 or 60 minutes) is sufficient to induce a strong increase of KCC2 expression (*Figure 6B*) associated with a higher phosphorylation of ERK1/2 (*Figure 6C*). These results suggest that the functional modifications obtained through the chronic protocol could be ascribed to a precocious and transient increment of KCC2.

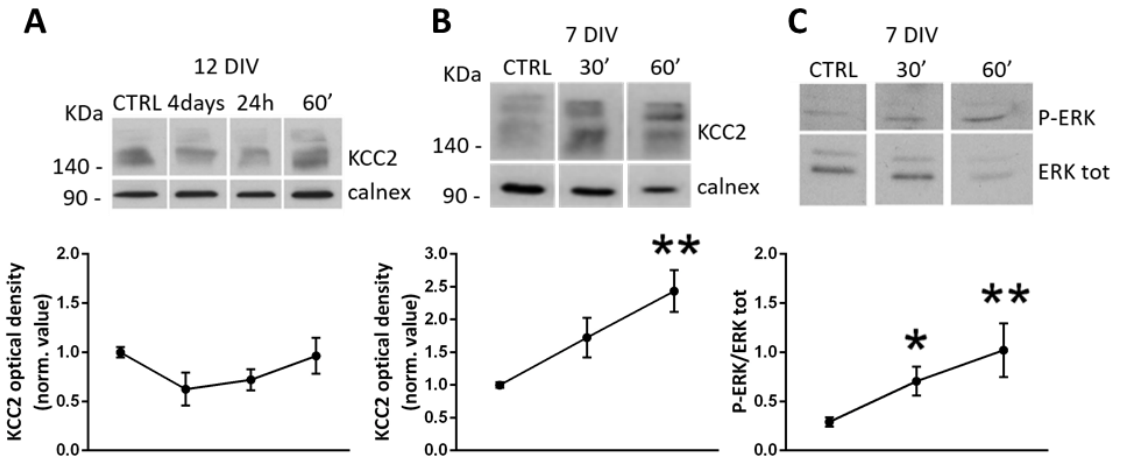


Figure 6. *Evaluation of KCC2 protein level upon different KU treatment paradigms.* ***A)*** *Western Blotting representative image and quantification of KCC2 expression in 12 DIV neurons treated with KU 1 µM for 4 days, 24 hours or 60 minutes, showing no differences between the experimental groups (Kruskal-Wallis Test followed by Dunn's Multiple Comparison Test, p=0,075. CTRL n=8, 4days n=6; 24h n=5; 60' n=2).* ***B)*** *Representative Western Blotting lanes and analysis of KCC2 levels upon KU acute treatment (30-60 minutes) in 7 DIV hippocampal cultures (One-way Anova followed by Holm-Sidak's Multiple Comparison Test, **p<0,01. CTRL n=7; 30' n=7; 60' n=7).* ***C)*** *Biochemical analysis of the amount of ERK phosphorylation in acutely KU-treated 7 DIV neurons compared to control (Kruskal-Wallis Test followed by Dunn's Multiple Comparison Test, *p<0,05 - **p<0,01. CTRL n=7; 30' n=7; 60' n=6).*

KU treatment promotes GABAergic development and reverts the E/I balance

Since Western Blotting data revealed that KU effects on KCC2 expression occur strictly during the early phases of neuronal development, we decided to modify our protocol treating neurons for one day at 5/6 DIV and to evaluate possible changes in the "excitatory-to-inhibitory switch of GABA" as a consequence of increased KCC2 levels (*Figure 7A*). Calcium imaging experiments performed in Fura-2AM loaded neurons showed that KU application for 24 hours accelerates GABAergic maturation, as indicated by a reduced percentage of cells responding to GABA with calcium transients (*Figure 7B*), suggesting an anticipated GABA-mediated hyperpolarizing action upon KU treatment. Surprisingly, we found that this effect is long-lasting, since even four days after KU application the excitatory-to-inhibitory switch of GABA remains accelerated with a reduced percentage of neurons responding to GABA stimuli by depolarization compared to control (*Figure 7C*). We also measured calcium transients evoked by KCl 50 mM application, in order to exclude the occurrence of possible changes in Voltage Operated Calcium Channels (VOCCs) expression and function upon KU delivery, but we did not find any changes between the two groups (*Figure 7D*). The evaluation of VOCCs in these kind of experiments is fundamental since their alterations could reflect variations in neuronal responses to GABA and, as a result, an altered estimation of the percentage of GABA responding neurons. Interestingly, Western Blotting data revealed increased KCC2 levels only one day but not 4 days after KU application (*Figure 7E*), suggesting that the acute boosting of KCC2 expression is sufficient to produce a long-lasting effect on GABA maturation.

To further demonstrate that the KU long-lasting effect on GABAergic development is exclusively determined by the acute boosting of KCC2 expression, we treated 6 DIV neurons with KU 1 µM for 24 hours, we co-applied VU 1 µM at 6 DIV and we continued VU application for the following two days (7-8 DIV) (*Figure 7F above*). VU inhibition of KCC2 activity blocks the KU-mediated long-lasting effect on GABA switch as indicated in (*Figure 7F bottom*).

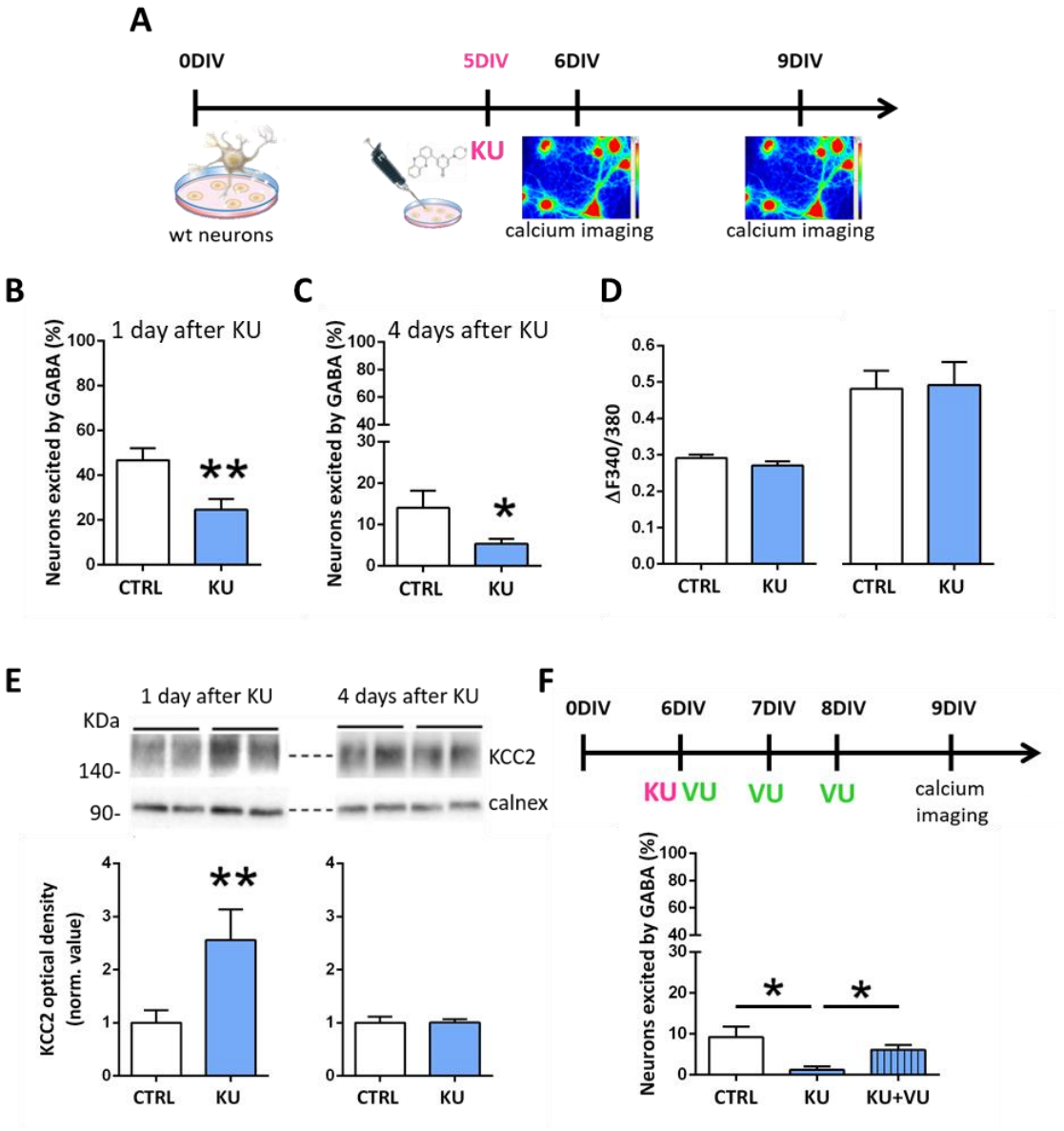


Figure 7. ***A)*** *Scheme of the experimental design for the evaluation of KU acute treatment induced effects on GABA switch.* ***B-C)*** *Quantification of the percentage of 6 DIV hippocampal neurons responding to GABA stimuli by depolarization one day (B) and four days (C) after KU 1 µM application with respect to controls (one-day treatment: Student's t-test, **p=0,004. CTRL n=395; KU=374. Four-days treatment: Student's t-test, *p=0,039. CTRL n=216; KU n=236).* ***D)*** *Analysis of calcium transients upon KCl 50 mM application in one day (left) and four days (right) treated-cells compared to controls display no changes in VOCC expression in all the experimental conditions (one-day treatment: Mann-Whitney Test, p=0.145. CTRL n=395; KU=374. Four-days treatment: Mann-Whitney test, p=0.777. CTRL n=216; KU n=236).* ***E)*** *Representative Western Blotting lanes and corresponding quantifications of KCC2 expression levels one day and four days after KU treatment* ***(****one-day treatment: Mann-Whitney Test, **p=0,005. CTRL n=7; KU n=11. Four-days treatment: Mann-Whitney test, p=0,918. CTRL n=7; KU n=11).* ***F)*** *Scheme showing the temporal line of KU and VU co-application experiment (above). Analysis of the excitatory-to-inhibitory switch of GABA reveals that VU administration prevents KU effects on GABAergic maturation (bottom) (Kruskal-Wallis Test followed by Dunn's Multiple Comparison Test, *p<0,05. CTRL n=204; KU n=163; KU+VU n=334).*

Our next goal was to investigate if the accelerated and potentiated GABAergic development induced by one-day treatment with KU could induce permanent changes of neuronal transmission. We therefore treated cells at 5/6 DIV and we measured excitatory and inhibitory synaptic activity in mature neurons (14 DIV) taking advantages of electrophysiological experiments (*Figure 8A*). Electrophysiological recordings revealed an increased inhibitory tone upon KU application, as indicated by the higher frequency (*Figure 8B - 8C left*) and amplitude (*Figure 8E*) of mIPSCs. The excitatory counterpart results instead decreased both in term of frequency (*Figure 8B - 8C right*) and amplitude (*Figure 8F*) of mEPSCs, leading to an E/I imbalance in favour of inhibition (*Figure 8D*). Accordingly, immunofluorescence analysis revealed increased mean size and mean intensity of vGAT-positive puncta (*Figure 9A-B-C*) and a reduction in these parameters in vGlut-positive puncta (*Figure 9A-D-E*).

These data suggest that KU acute application during development exerts long-lasting effects on synaptic transmission completely reverting the E/I balance in mature neurons.

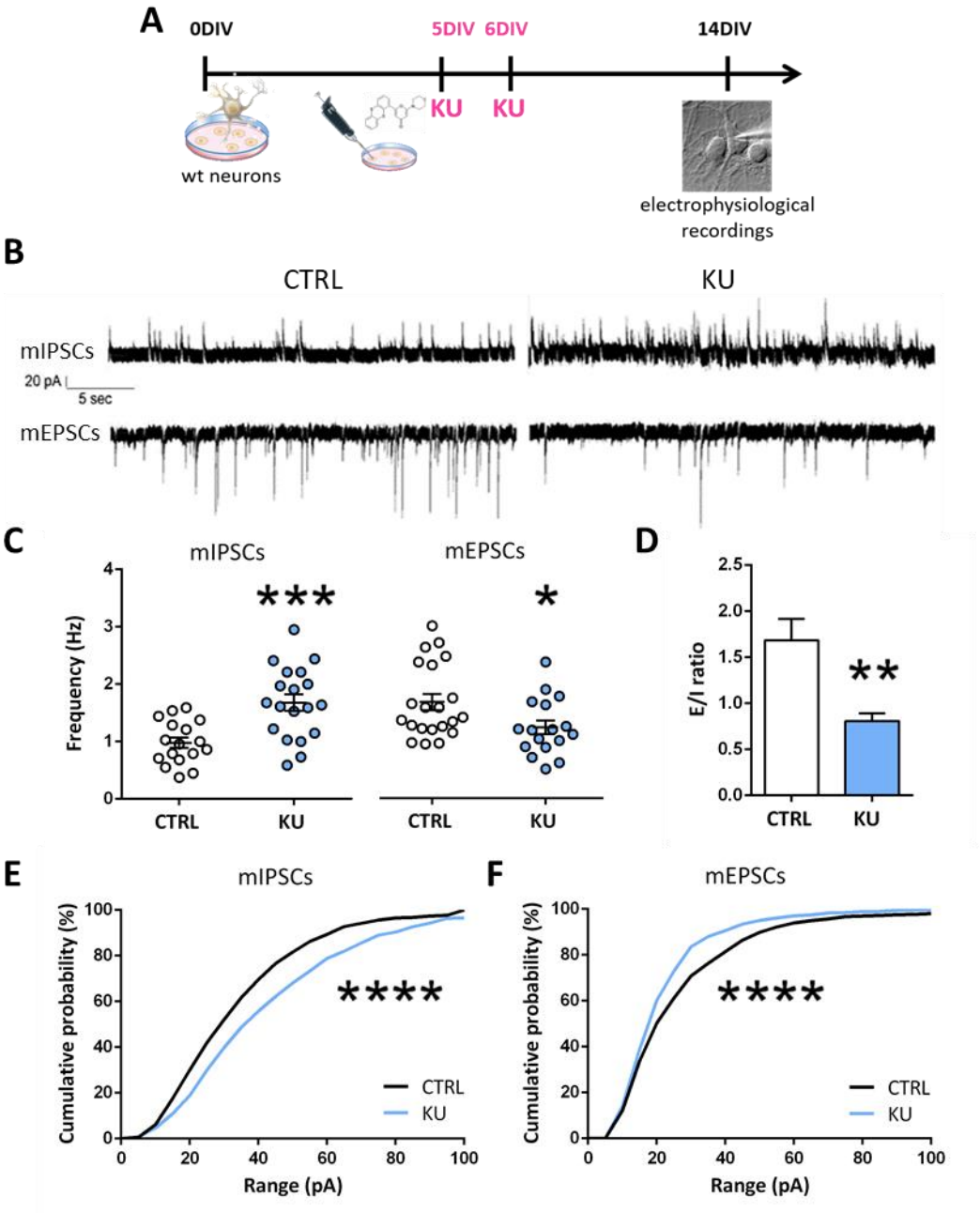


Figure 8. A) *Experimental time–line to measure changes in synaptic transmission upon KU acute treatment during development.* ***B)*** *Representative traces of mIPSCs and mEPSCs recorded in 14 DIV hippocampal neurons treated or not with KU 1 µM during development.* ***C)*** *Quantitative analysis of mIPSCs (left) and mEPSCs (right) frequency in KU-treated cells compared to control (mIPSCs: Student's t-test, ***p=0,0003. CTRL n=17; KU n=19. mEPSCs: Student's t-test, *p=0,026. CTRL n=21; KU=17).* ***D)*** *Electrophysiological quantification of the E/I ratio upon KU acute treatment (Student's t-test, **p=0,002. CTRL n=14; KU n=13).* ***E-F)*** *Analysis of mIPSCs and mEPSCs amplitude plotted as cumulative probability (mIPSCs: KS test, p<0,0001. CTRL n (events)=773; KU n (events)=823. mEPSCs: KS test, p<0,0001. CTRL n (events)=1378; KU n (events)=1037).*

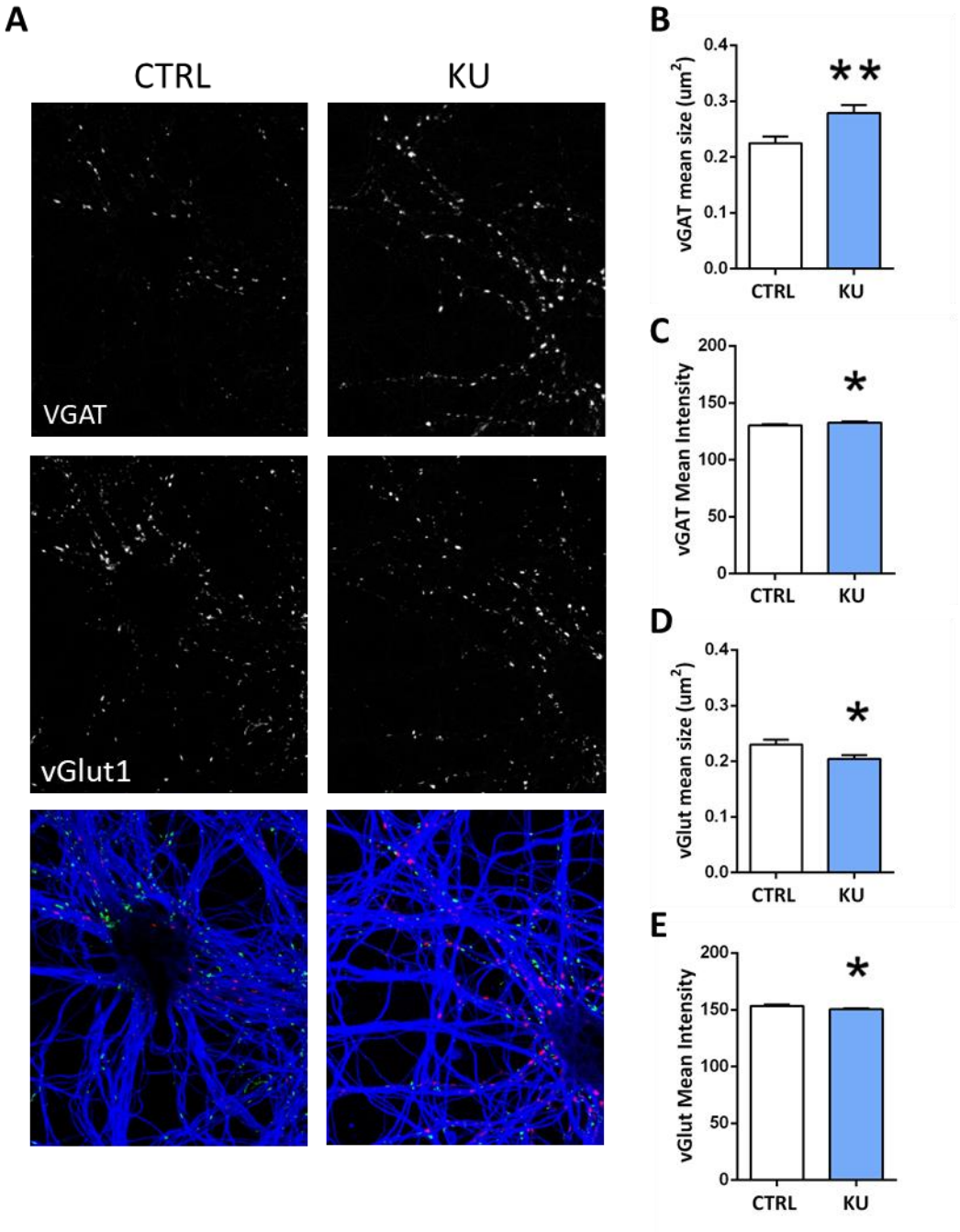


Figure 9. A) *Representative immunofluorescence images of 14 DIV hippocampal neurons labelled with the specific inhibitory and excitatory pre-synaptic markers, respectively vGAT and vGlut1, and with βIII-tubulin to highlight neuronal processes.* ***B-C)*** *Quantitative analysis of mean size (B) and mean intensity (C) of vGAT positive puncta in KU-treated neurons with respect to controls (mean size: Student's t-test **p= 0,004. Mean intensity: Student's t-test, *p=0,040. N=3 experiments, n=2 coverslips for each experiment).* ***D-E)*** *Quantitative analysis of mean size (D) and mean intensity (E) of vGlut positive puncta in KU-treated neurons vs controls (mean size: Student's t-test *p= 0,026. Mean intensity: Student's t-test, *p=0,021. N=3 experiments, n=2 coverslips for each experiment).*

KU counteracts the hyperexcitability induced in neurons

Whereas our ultimate goal in the study of KU effects is to exploit it as a new pharmacological tool in the treatment of disorders characterized by increased excitation and altered GABA maturation, we evaluated the possibility that the higher inhibition induced by KU treatment could counteract hyperexcitability of neuronal networks. To address this issue, we focused on hyperactivity processes acutely generated *in vitro* by exposing neurons to a Mg^{2+} free external medium [252, 253]. The Mg^{2+} removal indeed determines the unblock of NMDA glutamatergic receptors, which are normally silent at resting potential, since magnesium ions occupy the binding site deep inside the pore near the cytoplasmic side of the channel [254]. We therefore acutely treated cells with KU during development and we performed electrophysiological experiments at 14 DIV. We measured Multi Unit (MU) activity recording neurons in voltage-clamp mode and in cell attached configuration [255]. The analysis of this parameter allows to monitor the spiking activity of the recorded neuron as well as of its immediate neighbours and it is known to reflect the excitability of neuronal network [255]. As shown in *Figure 10A-B*, while the MU number was significantly increased in neurons exposed to the Mg^{2+}-free protocol with respect to controls (KRH), no statistically significant increment in MU frequency was observed after Mg^{2+} removal upon KU treatment. These data suggest that the higher GABAergic tone induced by KU is effective in counteracting the pharmacologically-mediated hyperexcitability and highlight KU as a new tool potentially exploitable in reverting pathological features of neurodevelopmental disorders characterized by increased excitation and/or reduced inhibition.

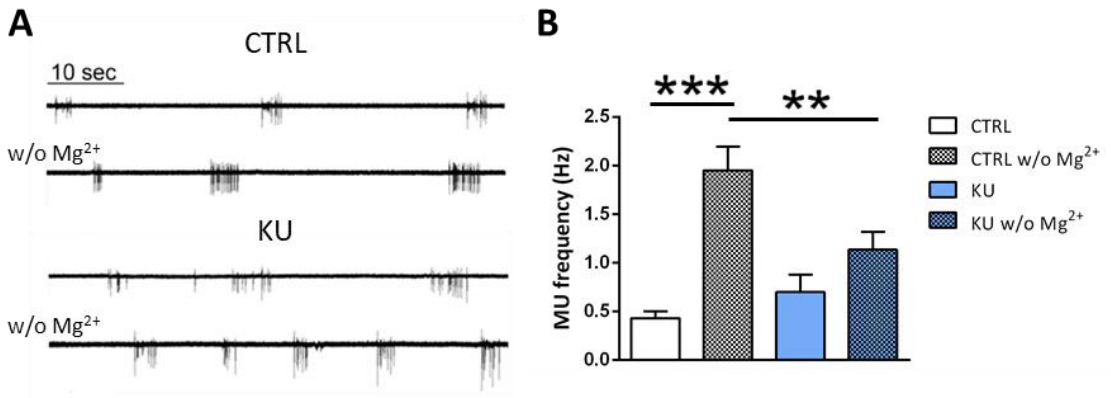


Figure 10. A) *Representative electrophysiological traces of Multi-Unit (MU) activity (i.e. spiking activity) recorded in voltage-clamp in cell attached configuration in 14 DIV hippocampal cultured neurons, treated or not with KU during development, before and after the Mg^{2+} removal from the external solution.* ***B)*** *Quantitative analysis of MU frequency reveals potentiated activity in control cultures but not in KU treated cells during development, after the application of the hyperexcitability paradigm (One-Way Anova followed by Holm-Sidak's Multiple Comparison Test: ***p<0,001 - **p<0,01. CTRL n=34; CTRL w/o Mg^{2+} n=28; KU n=21; KU w/o Mg^{2+} n=27).*

Assessment of the possible molecular mechanisms underlying KU effects

KU is effective in enhancing KCC2 levels even *in vivo*

To further demonstrate the pharmacological applicability of KU in neurological disorders, we investigated KU effectiveness *in vivo.* To this purpose, we performed intracerebroventricular injections (ICV) of 3 µl of KU 10 µM in the right ventricle of post-natal day 4 (P4) wild-type mice and we quantified KCC2 expression 24 hours later. We chose this administration paradigm since KU displays a very limited solubility and it is not able to cross the blood-brain barrier [235]. Western Blotting data showed a higher expression of KCC2 in brains of KU-treated mice compared to controls (DMSO-injected mice), indicating KU effectiveness also *in vivo (Figure 11).*

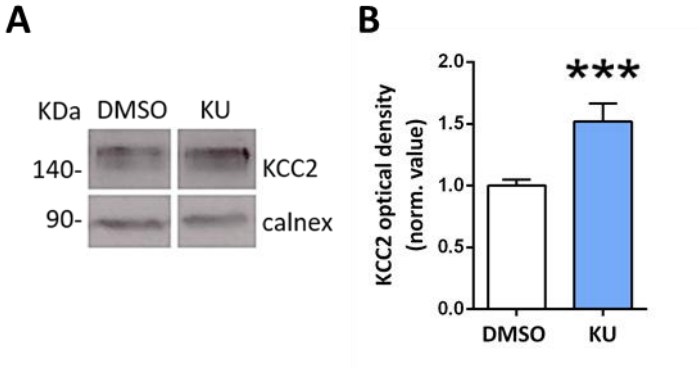


Figure 11. A-B) *Representative Western Blotting lanes and quantitative analysis of KCC2 expression levels measured in P5 wild-type mice 24 hours after a single intracerebroventricular injections of KU 10µM (3 µl) in the right ventricle (Mann-Whitney Test, p=0,0008. Number of animals=12 per group).*

Since in literature it is known that the concentration of KU 2 µM can inhibit autophagosome formation *in vitro* [243], we evaluated the impact of our treatment (KU 10 µM - ICV) on basal autophagy *in vivo*. This is an extremely important point

considering that autophagy processes are crucial during neurodevelopment, especially for the formation of correct synaptic contacts, and their dysregulation plays a crucial role in neurological disorders [8, 256, 257]. To address this issue, we measured the expression levels of a pivotal marker of autophagy, the microtubule-associated protein 1 light chain 3 BII isoform (LC3-BII) [258, 259], in tissues explanted from KU-injected pups. As shown in *Figure 11A-B*, one day after KU *in vivo* administration, LC3-BII expression remains unaffected. We also evaluated Heat Shock Protein Family A (Hsp70) Member 8 (HSPA8) levels since it is an ubiquitous molecular chaperone known to play an important role in protein folding and degradation, stress response, endosomal micro-autophagy, and chaperone-mediated autophagy [260, 261]. However, we did not find any changes after KU application *Figure 11A-C*. Thus, in our experimental conditions, KU does not seem to impact autophagy pathways.

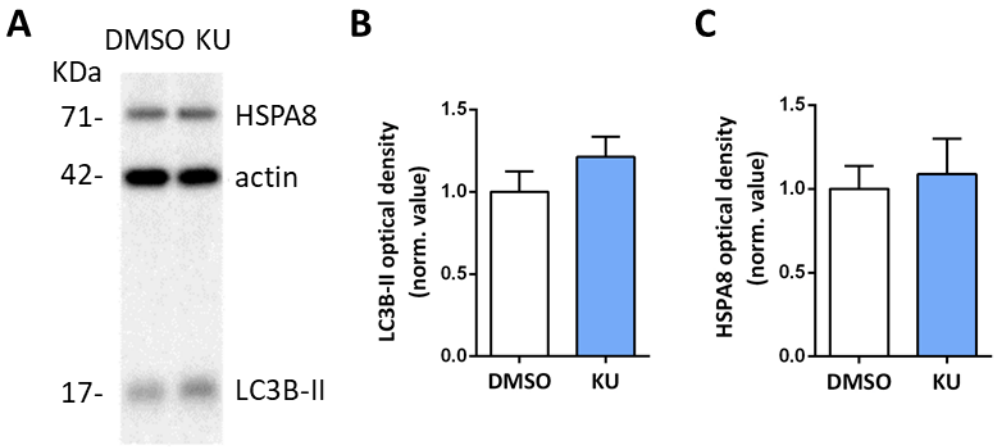


Figure 12. A) *Representative Western Blotting lanes of the autophagy marker LC3B-II, the endosomal micro-autophagy and chaperone-mediated autophagy marker HSP8A, and the house-keeping protein actin.* ***B-C)*** *Quantitative analyses of LC3B-II (B) and HSPA8 (C) expression in KU-injected P5 pups' brains with respect to controls (LC3B-II: Student's t-test, p=0,237. HSPA8: Student's t-test, p=0,642. Number of animals=12 per group).*

KU promotes KCC2 expression through Egr4 activation of *Kcc2b* promoter

We then wanted to define the molecular mechanisms responsible of KU effects. In literature is known that KCC2 expression during development is tightly regulated by Egr4 transcription factor which, once activated by ERK protein, promotes the transcription of *Kcc2* gene (*Figure 13A*) [50, 262]. In our previous study we found increased KCC2 levels correlated to a higher ERK phosphorylation in *Atm* Het mice [220], so we hypothesized a possible involvement of Egr4 pathway in ATM regulation of KCC2 expression. To better investigate this hypothesis, we took advantage of a construct including the -309/+42 region of the *Kcc2b* mouse promoter, which contains exclusively the Egr4 consensus sequence [262], followed by the NanoLuc luciferase gene reporter. Thus, we transfected 5 DIV neurons with the construct, we applied KU 24 hours later (6 DIV), and we measured the NanoLuc and Luc2 luciferase activity by a Dual-Luciferase Reporter Assay System one day after KU application (7 DIV). As shown in *Figure 13B*, we measured an increased NanoLuc/Luc2 signal in KU treated cultures compared to control, indicating that ATM involvement in GABAergic development is linked to a transcriptional mechanism involving the Egr4-dependent activation of *Kcc2b* promoter.

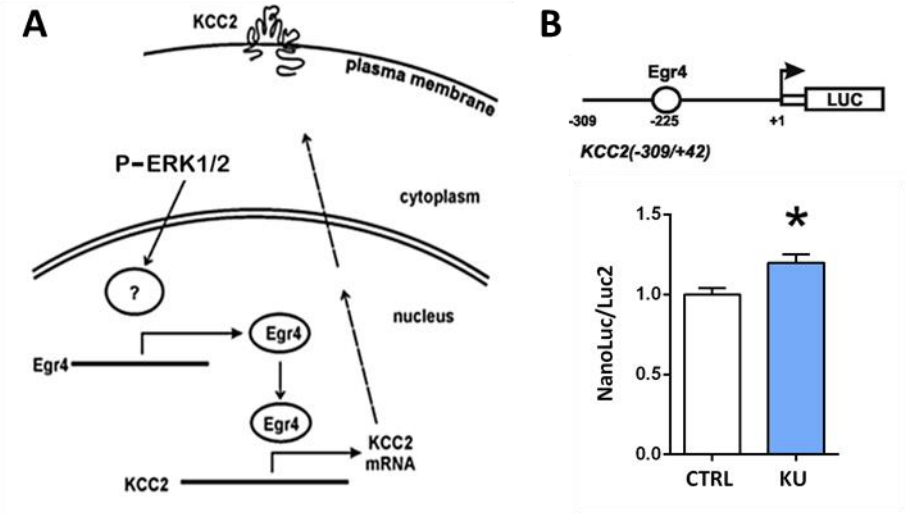


Figure 13. A) *Simplified schematic diagram of the signal transduction cascade involved in Egr4-induced KCC2 expression. Modified from Ludwig, et al 2001.* ***B)*** *Scheme of the transfected construct to evaluate Egr4 activity on Kcc2b promoter through Luciferase assay (above). Quantitative analysis of NanoLuc/Luc2 luciferase activity in 7 DIV transfected cells upon KU application for one day (Student's t-test, *p=0,019. CTRL n=5; KU n=8) (below).*

Coherently, a significantly higher Egr4-dependent activity of *Kcc2b* promoter was found in *Atm* Het neurons (7 DIV) respect to WT (*Figure 14A*), indicating Egr4 pathway implication also in the genetic reduction of ATM. Surprisingly, *Atm* knockout (KO) neurons display a lower Egr4 activity respect to WT even in presence of an increased KCC2 expression in hippocampal tissues (*Figure 14B*). These results indicate the presence of an additional mechanism linked to ATM that converge in a potentiated KCC2 expression.

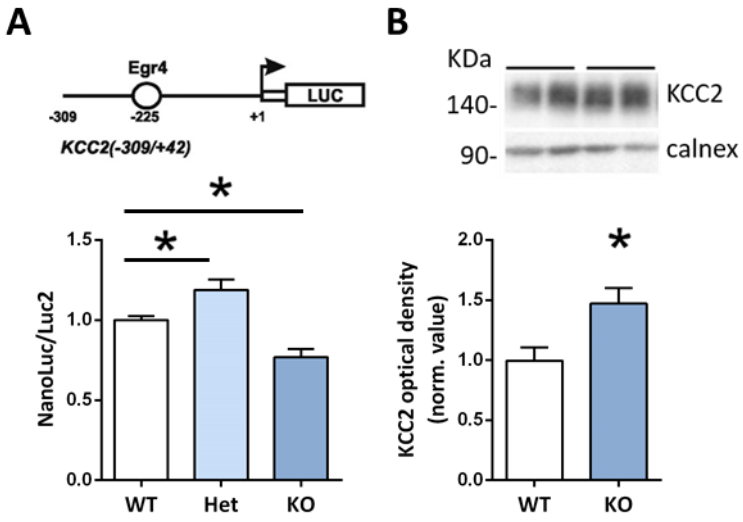


Figure 14. A) *Luciferase quantitative analysis of Egr4 activity on Kcc2b promoter in 7 DIV hippocampal neurons obtained from Atm WT/Het/KO embryos (One-Way Anova followed by Dunnett's Multiple Comparisons Test, *p<0,05. WT n=6, Het n=7, KO n=7).* ***B)*** *Western Blotting representative image and quantification of KCC2 expression levels in Atm KO hippocampi with respect to WT (Student's t-test, *p=0,032. WT n=4; KO n=4).*

KU enhances *Mecp2* transcription

To define the mechanism responsible for the increased KCC2 expression in *Atm* KO mice, we wondered if other transcriptional pathways could operate in parallel and independently to Egr4. Several studies point out the direct regulation of KCC2 expression via Methyl-CpG-binding protein 2 (MeCP2) activity both in human and in mice [60, 69, 74]. Notably, it has been demonstrated that Mecp2, occupying the repressor element-1 (RE-1) sites in the *Kcc2* gene, prevents REST binding and so the inhibition of KCC2 expression [60]. Thus, we evaluated Mecp2 levels in both genetic and pharmacological mouse models of ATM depletion. Surprisingly, Western Blotting data revealed higher levels of Mecp2 not only in hippocampal tissues extracted from *Atm* KO mice (*Figure 15A*) but also in *Atm* Het and in KU-injected (3 μL of KU 10 μM) mice with respect to controls (*Figure 15B-C*).

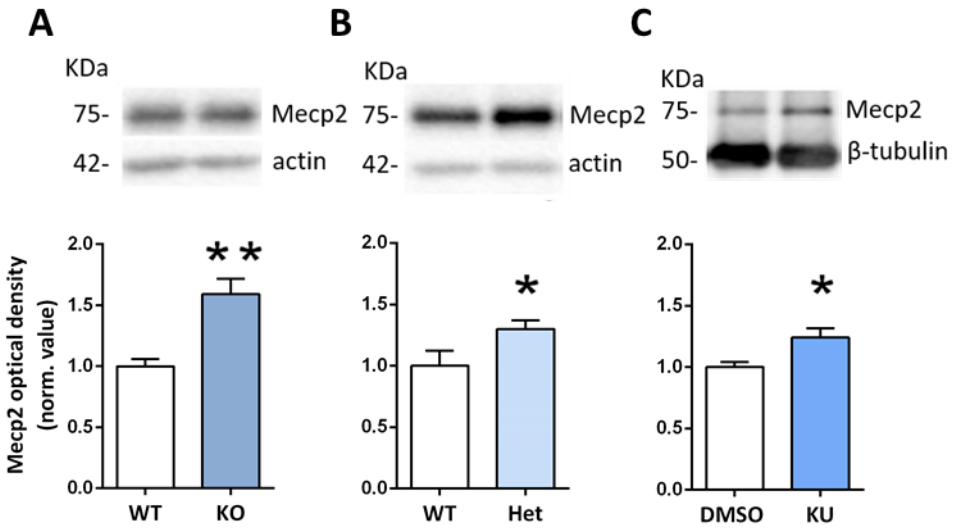


Figure 15. *Representative images and corresponding analyses of Mecp2 expression in brains of both genetic and pharmacological mouse models of Atm depletion.* ***A)*** *Mecp2 increased level in Atm KO hippocampi compared to WT (Student's t-test, **p=0,006. WT n=4; KO n=5).* ***B)*** *Mecp2 increment in Atm Het hippocampi respect to the WT counterpart (Student's t-test, *p=0,040. WT n=5; Het n=10).* ***C)*** *P5 KU-injected pups' brains display higher Mecp2 expression compared to DMSO controls (Student's t-test, *p=0,011. DMSO n=14; KU n=15).*

In parallel, immunofluorescence experiments carried out on hippocampal slices confirmed increased Mecp2 expression after KU ICV injection, as indicated by confocal analysis of Mecp2 integrated density (*Figure 16A-B*).

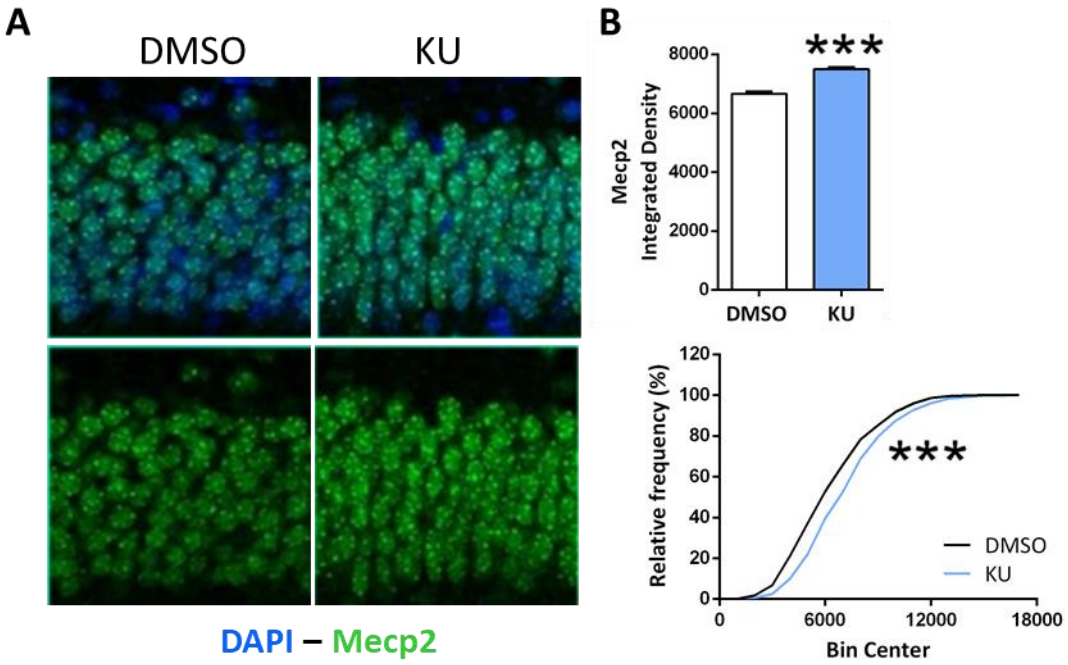


Figure 16. A) *Representative confocal immunofluorescence images of hippocampal slices obtained from KU-injected mice scarified 24h after KU injection, labelled with Mecp2 antibody (green) and DAPI (blue) to identify the nuclei.* ***B)*** *Quantification of integrated density of fluorescence for Mecp2 signal in DAPI positive nuclei plotted in column graph (above) and as relative frequency percentage (below) (Mann-Whitney Test, ***p<0,001. KS test: ***p<0,001. Number of animals: DMSO=4 vs KU=4; number of slices: DMSO=12 vs KU=12; number of images quantified per slices=6).*

As shown in *Figure 17*, qRT-PCR analysis of *Mecp2*-mRNA levels revealed that *Mecp2* transcription is transiently potentiated 30 and 60 minutes after KU application, suggesting that ATM activity impacts Mecp2 expression at transcriptional level. All together these results demonstrate that KCC2 expression is finely modulated by ATM kinase activity through Egr4 and Mecp2 pathways which occur independently.

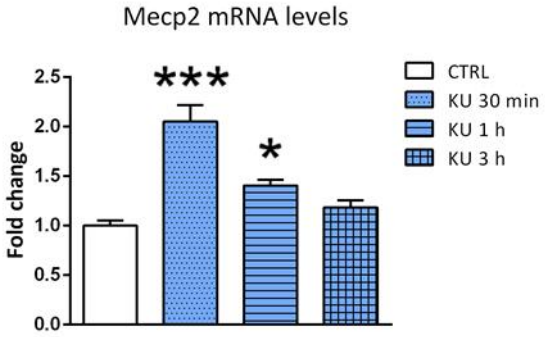


Figure 17. *Quantification of Mecp2 mRNA levels by real time PCR experiments in hippocampal cultured neurons treated with KU or DMSO as control for different duration (Kruskal-Wallis followed by Dunn's Multiple Comparisons Test: ***p<0,001 - *p<0,05. CTRL n=7; KU 30 min n=5; KU 1h n=5; KU 3h n=5).*

Evaluation of KU applicability to rescue neuronal dysfunctions in the mouse model of Rett syndrome

Genetic lack of Mecp2 results in ATM increased expression

To further confirm the link between ATM and Mecp2 we took advantages of *Mecp2*$^{y/-}$ mice, the mouse model of Rett syndrome. We evaluated ATM expression in P6-7 *Mecp2*$^{y/-}$ mice compared to sex matched wild-type (WT). Surprisingly, Western blotting experiments showed an increased amount of ATM protein (*Figure 18A-B*) in association with reduced KCC2 levels (*Figure 18A-C*), as already described in literature [60], in hippocampal tissues extracted from *Mecp2*-deficient P6 young mice with respect to controls.

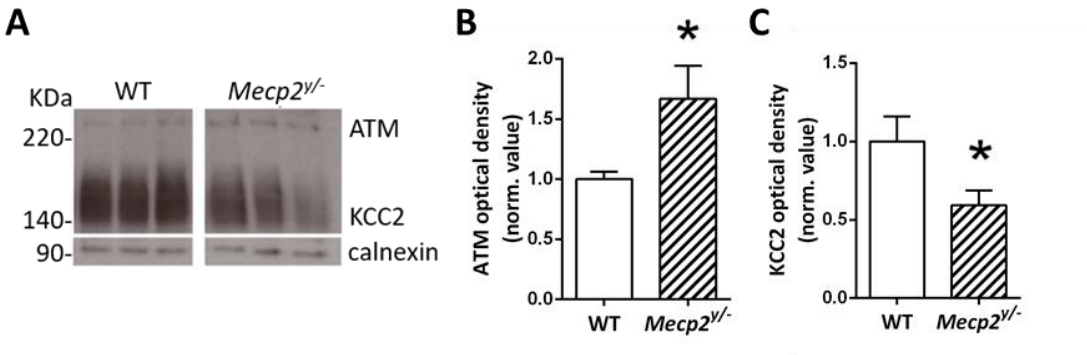


Figure 18. A) *Representative Western Blotting lanes showing ATM increased expression related to KCC2 low levels in Mecp2-deficient mice and the house-keeping protein calnexin.* ***B)*** *Quantification of ATM expression (Mann-Whitney Test, *p=0,042. WT n=9; Mecp2*$^{y/-}$ *n=13).* ***C)*** *Quantitative analysis of KCC2 levels (Mann-Whitney Test, *p=0,026. WT n=6; Mecp2*$^{y/-}$ *n=6).*

KU rescues functional defects in *Mecp2*$^{y/-}$ neurons

After demonstrating the capability of KU treatment in modulating KCC2 expression and E/I balance, our next goal was to test its efficacy in rescuing pathological phenotypes of neurodevelopmental disorders. Since in literature is widely accepted that Rett syndrome is characterized by a delayed GABAergic maturation, low KCC2 levels and E/I imbalance [60, 69, 71], we evaluated the possibility to exploit KU action to ameliorate neuronal defects in *Mecp2*$^{y/-}$ mice. In addition, we unveiled for the first time that *Mecp2* genetic ablation results in increased ATM expression in mice, suggesting a possible ATM implication in Rett aethiopathology. We therefore treated *Mecp2*$^{y/-}$ neuronal primary cultures with the ATM inhibitor KU during development (DIV 7/8) and we performed calcium imaging experiments and electrophysiological recordings to evaluate respectively excitatory-to-inhibitory switch of GABA and neuronal activity (*Figure 19A*). Calcium imaging data showed a significantly higher percentage of neurons responding to GABA stimuli with calcium transients in *Mecp2*$^{y/-}$ preparations compared to wild-type (WT), indicating a still depolarizing action of GABA and a delayed GABA switch in *Mecp2*-deficient mice. This developmental retardation is completely normalized through KU application one day prior to calcium imaging experiments (*Figure 19B*). To exclude VOCC changes upon KU treatment in *Mecp2*$^{y/-}$ cells, we evaluated KCl-induced calcium responses, but we observed no changing in amplitude of induced-calcium transients in all the experimental conditions (*Figure 19C*). As displayed in *Figure 19D*, we also found reduced calcium peaks induced by GABA application in *Mecp2*$^{y/-}$ neurons compared to WT, indicating a significant alteration in GABA receptor expression, which is restored by KU treatment.

Finally, the hyperexcitability induced by exposing neurons to a Mg^{2+}-free external medium was observed only in 14 DIV *Mecp2*$^{y/-}$ neuronal cultures, as shown in *Figure 19E-F*, by the significantly higher spiking activity (MU frequency) with respect to control (KRH external solution). No significant differences in terms of firing frequency could instead be detected in *Mecp2*$^{y/-}$ cells treated with KU during development and then exposed to the hyperexcitability paradigm at 14 DIV (*Figure 19E-F)*.

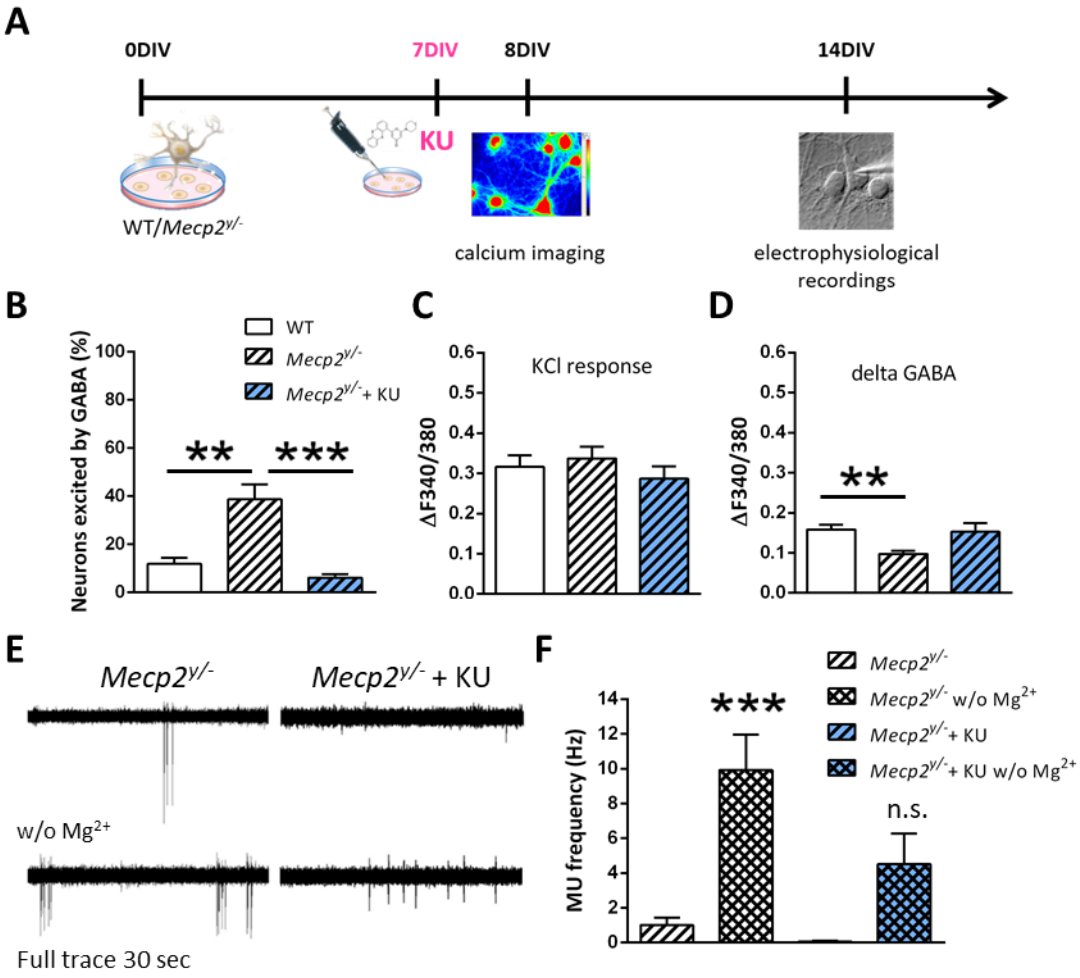


Figure 19. A) *Scheme showing the experimental time-line of KU treatment and functional experiments carried-out in Mecp2$^{y/-}$ neurons.* ***B)*** *Calcium imaging quantification of the percentage of 8 DIV neurons responding to GABA stimuli by depolarization (Kruskal-Wallis followed by Dunn's Multiple Comparisons Test, **p<0,01 - ***p<0,001. WT n=227; Mecp2$^{y/-}$ n=93; Mecp2$^{y/-}$ + KU n=215).* **C)** *Analysis of calcium transients evoked by KCl application shows no changes in VOCC expression (Kruskal-Wallis followed by Dunn's Multiple Comparisons Test, p=0,292. WT n=227; Mecp2$^{y/-}$ n=93; Mecp2$^{y/-}$ + KU n=215).* ***D)*** *Calcium transients upon GABA 100 µM stimulation indicate a lower amount of GABA receptors expression in Mecp2$^{y/-}$ cells which is rescued by KU treatment during development (Kruskal-Wallis followed by Dunn's Multiple Comparisons Test, **p<0,01. WT n=58; Mecp2$^{y/-}$ n=40; Mecp2$^{y/-}$+KU n=34).* ***E)*** *Electrophysiological traces of Multi-Unit (MU) activity (i.e. spiking activity) recorded in voltage-clamp in cell attached configuration in 14 DIV Mecp2$^{y/-}$ hippocampal cultured neurons, treated or not with KU during development, before and after the Mg^{2+} removal from the external solution.* ***F)*** *Quantification of MU frequency displays that Mecp2$^{y/-}$+KU neurons are resistant in generating the pharmacological hyperexcitability induced by Mg^{2+} removal (One-Way Anova followed by Sidak's Multiple Comparisons Test, ***p<0,001. Mecp2$^{y/-}$ n=17; Mecp2$^{y/-}$ w/o Mg^{2+} n=16; Mecp2$^{y/-}$+KU n=18; Mecp2$^{y/-}$+KU w/o Mg^{2+} n=14).*

We then investigated the molecular mechanism underlying KU rescuing effect of neurological defects in *Mecp2*$^{y/-}$ neuronal cultures. We took advantage of Luciferase assay and we found a potentiated Egr4 activity on *Kcc2b* promoter (*Figure 20A*) in accordance to normalized KCC2 expression levels (*Figure 20B*) in *Mecp2*$^{y/-}$ cells treated with KU.

Altogether these data strongly suggest that KU tuning of ATM activity could represent a novel tool to treat developmental retardation in the mouse model of Rett syndrome.

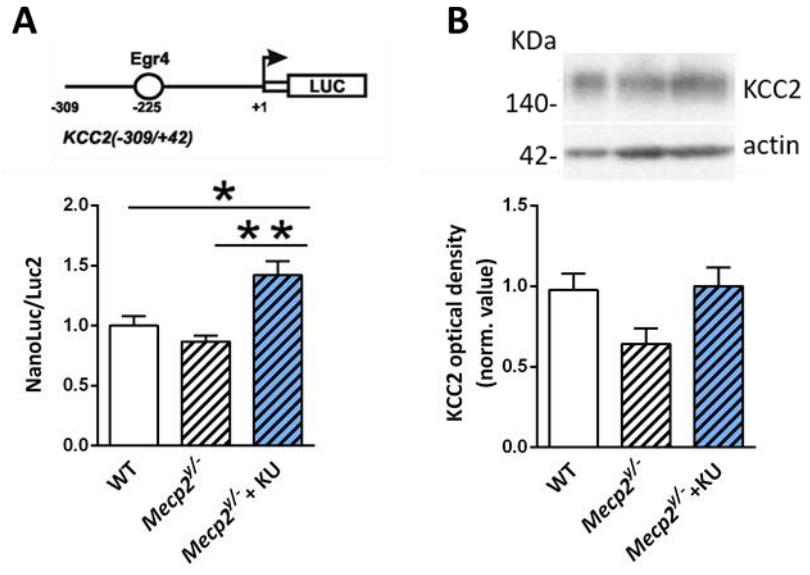


Figure 20. A) *Luciferase analysis indicates that KU treatment potentiates Egr4 activity on Kcc2b promoter in Mecp2*$^{y/-}$ *neurons (Ordinary One-way Anova followed by Tukey's multiple comparison test, *p<0,05 - **p<0,01. Number of samples (isolated embryos) WT n=6; Mecp2*$^{y/-}$ *n=4; Mecp2*$^{y/-}$*+KU n=4).* ***B)*** *Biochemical analysis shows KU rescuing effect of KCC2 expression in Mecp2*$^{y/-}$ *treated neurons (One-Way Anova, *p=0,048. WT n=8; Mecp2*$^{y/-}$ *n=8; Mecp2*$^{y/-}$*+KU n=9).*

DISCUSSION

In this study we highlight the importance of ATM kinase activity in the physiological development of central neurons, especially, its key role in regulating GABAergic maturation, inhibitory transmission and in maintaining the proper excitatory/inhibitory balance. We reveal ATM dysregulation as a novel aethiopathological factor responsible for neuronal dysfunctions in neurodevelopmental disorders, i.e. Rett syndrome. Notably, we demonstrate a completely novel application of the ATM inhibitor KU, a compound already used in oncological pre-clinical research, as an innovative pharmacological tool for the treatment of neurodevelopmental disorders characterized by altered GABAergic maturation, poor inhibition and neuronal hyperexcitability.

The maintenance of a proper balance between excitatory and inhibitory neurotransmission is crucial for a the correct brain development, function and plasticity [9, 13]. Thus, alterations in GABA developmental steps may result in pathological conditions such as epilepsy, autism and schizophrenia [26, 263]. A fundamental process in GABAergic maturation is the so called "excitatory-to-inhibitory switch of GABA", the postnatal transition of GABA transmission from excitatory to inhibitory [38]. In our previous study we demonstrated an anticipated GABA switch and an increased inhibitory tone, associated to higher KCC2 expression levels, in hippocampal neurons established from ATM heterozygous embryos [220], suggesting ATM role in controlling neuronal development and inhibitory transmission. The aim of this thesis was to investigate the possibility to mimic, by a pharmacological intervention, the accelerated GABAergic development and potentiated inhibitory tone typical of the ATM heterozygous phenotype, to propose a new tool for the treatment of neurological disorders associated with developmental alterations and neuronal hyperexcitability.

To verify our hypothesis, we tested the effectiveness of KU, a potent inhibitor of ATM kinase activity largely studied in oncology because of its ability to inhibit cancer proliferation by inducing apoptosis [227, 230]. We first identified 1 μM as the lowest concentration of KU able to inhibit ATM but free from inducing toxic effects in neuronal cultures. This is in line with several studies which indicate that i) KU 5 μM is the highest non-toxic drug concentrations linked to a cell viability above 85% [242]; ii) KU 2 μM does not affect cell survival [243]. Moreover, KU exhibits a potent specificity for ATM

kinase with at least a 100-fold differential in selectivity in a counter-screening of KU against other members of the PIKK family. Moreover, several studies show that KU concentrations up to 10,000 times higher than 1 µM display a good selectivity for ATM kinase pathways, i.e. KU 10 mM has no significant effects on unspecific pathways such as the CREB transcriptional basal activity [244]. In contrast, in an *in vitro* study Farkas and colleagues demonstrated that KU inhibits autophagy at concentration of 2 µM. They excluded ATM kinase involvement in autophagy pathway and they identified Vps34 as a direct target of KU [243]. Since we used KU 10 µM for *in vivo* experiments, we verified the possible KU-induced inhibition of basal autophagy in KU injected mice. We checked the expression levels of the pivotal autophagy marker, the microtubule-associated protein 1 light chain 3 BII isoform (LC3-BII) [258, 259], and the Heat Shock Protein Family A (Hsp70) Member 8 (HSPA8), an ubiquitous molecular chaperone known to play an important role in protein folding and degradation, endosomal micro-autophagy and chaperone-mediated autophagy [260, 261]. We did not find any changes in the expression of these proteins even if the KU concentration that we applied *in vivo* is higher than the one implicated in the autophagy pathway *in vitro*. We explain these results by the evidence that i) once injected in the ventricle, KU gets diluted so the final effective concentration is much lower than 10 µM; ii) starting by the same drug concentration, KU will differently impact neuronal function in an *in vivo* protocol, in which the complexity of the brain structure is maintained, compared to an *in vitro* simplified model; iii) KU inhibition of autophagosome formation has been demonstrated in an autophagy-activated paradigm whereas we evaluate KU effects in steady-state neurons or mice. Thus, our results well combine with the literature.

In our previous publication, we demonstrated a precocious development of inhibitory system linked to increased KCC2 expression and higher inhibitory tone in *Atm* heterozygous mice [220]. Taking advantages of the ATM kinase inhibitor KU, in the current study we highlight the importance of ATM kinase activity in physiological brain development and in neurodevelopmental disorders aetiology. Results collected in this thesis provide additional and stronger evidence of the direct link between ATM activity – KCC2 expression and inhibition levels, corroborating and expanding what we showed in Pizzamiglio et al, 2016 [220]. Moreover, these data reveal that the tuning of ATM

activity impacts KCC2 expression only in a restricted temporal window during development, which can be defined as “critical period for KCC2 production”. At this stage of maturation, the system is particularly sensitive to changes, indeed a short-term ATM inhibition, by accelerating the excitatory to inhibitory switch of GABA, induces long-lasting effects on neuronal transmission. Coherently, it is widely accepted that a perturbation of neuronal development, even if of short duration, can deeply and permanently modify neuronal function [264-266].

Importantly, our results disclose the molecular mechanisms underlying ATM essential role in the maturation of GABAergic system. Exploiting both the pharmacological and the genetic models of ATM deficiency, we demonstrated that ATM regulates *Kcc2* transcription through two different and independent pathways involving the transcription factor Egr4, and the epigenetic regulator Mecp2. Egr4 directly promotes KCC2 expression by binding *Kcc2b* promoter [50, 262], whereas Mecp2, occupying the repressor element-1 (RE-1) sites in the *Kcc2* gene, prevents REST binding and inhibition of KCC2 expression [60]. In presence of reduced but still present ATM activity, i.e. KU-treated cells/mice and in *Atm*-Het neurons/mice, both these two mechanisms occur leading to higher KCC2 levels, as indicated by the increased Egr4 activity on *Kcc2b* promoter and the higher Mecp2 expression. In *Atm*-KO, where ATM is completely absent, the Egr4 activity is reduced and the higher KCC2 signal is exclusively linked to the increased Mecp2 levels. *Vice versa*, in *Mecp2-null* brains, in which we found higher ATM and reduced KCC2 levels, KU administration normalizes KCC2 expression potentiating Egr4 activity. Thus, variations in ATM levels reflect opposed KCC2 expression and behaviour in terms of GABAergic development based on Egr4 and Mecp2 dependent-mechanisms.

The results collected in this work acquire relevance since ATM emerges as a new potential target to restore the proper excitatory/inhibitory transmission in conditions associated to hyperexcitability and delayed GABAergic maturation. In fact, we demonstrated that inhibition of ATM activity in *Mecp2*$^{y/-}$ neurons, the mouse model of Rett syndrome, rescues abnormal GABAergic development and normalizes E/I balance and neuronal hyperexcitability. As in the case of Rett syndrome, a large number of

psychiatric and neurodevelopmental disorders are mainly characterized by E/I imbalance and failure of inhibition, as indicated in: i) *Oxt*$^{-/-}$ mouse model of autism spectrum disorder [80, 267]; ii) *Scn1a*$^{+/-}$ model of a severe epilepsy of infancy, the Dravet syndrome [77]; iii) FMRP mice, the mouse model of the Fragile-X syndrome associated to mental retardation [268]; iv) heterozygous *reeler* mice, one of the mouse models of schizophrenia [269]; v) *Lgdel*$^{+/-}$ mouse model of 22q11.2 deletion syndrome (22q11.2 DS) associated to autism, schizophrenia and cognitive impairments [270]; and so on. Thus, ATM tuning could be tested as an innovative treatment in a plethora of neurological disorders.

Also, our work perfectly fit with recent studies which highlight the role of proteins involved in DNA double-strand breaks (DSBs) machinery as fundamental regulators of neuronal function [104, 106]. In particular, it has been demonstrated that diverse paradigms of neuronal stimulation, such as mice exposure to physiological learning behaviours, determine the formation of DSBs. These activity-dependent DSBs are restricted to few loci through the entire genome enriched for the early response genes *Fos*, *Npas4*, *Egr1*, and *Nr4a*, whose induction regulates experience-driven changes to synapses including synaptogenesis, neurite outgrowth, synaptic strength and maturation [107]. This process seems to be crucial since alterations of this pathway result in cognitive disabilities [112]. In this scenario, our present results, provide further proofs that defects in DSBs related proteins may generate neurological disfunctions and point out that a better understanding of the mechanisms underlying these alterations will be of enormous significance.

Finally, this study demonstrates for the first time that among the pathological modifications occurred in the mouse model of Rett Syndrome, higher ATM levels contribute to the generation of the altered neuronal phenotype. ATM signalling has been found consistently elevated also in cells and brains derived from the mouse model of Huntington disease (HD) and in HD patients' brain tissues [118]. Notably, the genetic reduction of *Atm,* reaching by crossing the murine *Atm* heterozygous null allele onto mice expressing full-length human mutant Huntingtin, ameliorates multiple behavioural deficits and partially improved HD neuropathology [118]. Also, in two mouse models of

HD, it has been found a reduced KCC2 expression and then a still excitatory action of GABA in mature neurons [271], that perfectly fit with the high amount of ATM protein. This alteration in inhibitory maturation seems to underlie cognitive defects of HD mouse model since restoring proper GABAergic function rescues memory deficits [271]. On the other hand, loss-of function mutations in *ATM* gene, resulting in ATM deficiency, have been associated to cognitive impairments among which defects of attention, verbal memory, non-verbal memory, late learning and fluency of speech [142]. Coherently, Nora D. Volkow and colleagues demonstrated by positron emission tomography a reduced glucose metabolism in some brain regions, including the hippocampus, in homozygous and heterozygous patients compared to healthy controls [143]. This evidence suggests that both increase and decrease of ATM levels correlate with brain changes. Thus, the maintenance of a balanced ATM kinase activity is necessary for the correct brain maturation and function. Further experiments are needed to better investigate the involvement of ATM in the aetiopathogenesis of other neurological diseases such as autism spectrum disorders and epilepsy, in which low KCC2 levels and hyperexcitability are common pathological features. Mostly, the inhibition of ATM activity in neurological diseases through KU offers, as an example of "drug repositioning", the big benefit to shorten time of drug characterization and to exploit an old drug in a new field.

The results collected in this thesis strongly support our idea to collocate ATM as a new key protein responsible for the physiological development of central neurons and highlight ATM inhibition as a prospective therapeutic tool in neurodevelopmental disorders.

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