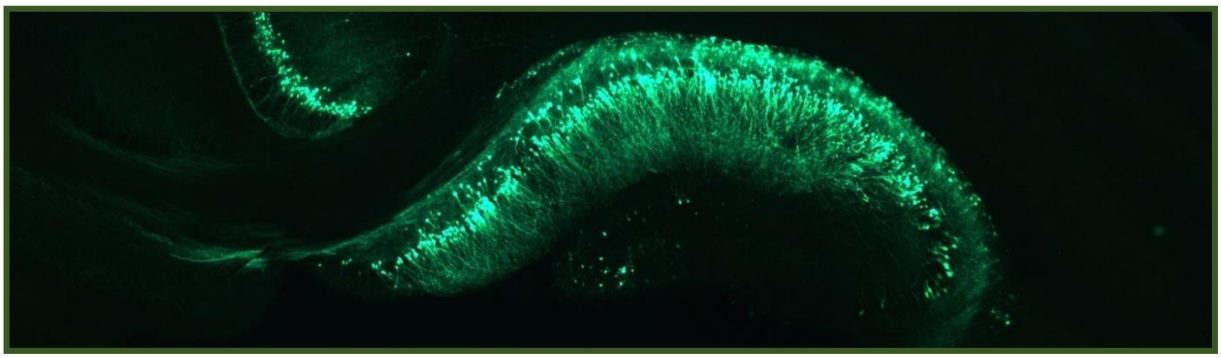

PhD Course in Neuroscience
Curriculum in Neuroscience and Brain Technologies
Cycle XXXIII

Long-Term Consequences of Early-in-Life Genetic and Pharmacological Interventions In Down Syndrome Mice

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Science is a way of thinking

much more than it is a body of knowledge.

Carl Sagan

Contents

Abstract

Down syndrome (DS) is the leading cause of genetically-defined intellectual disability. Additionally, DS individuals often present with increased susceptibility to epileptic seizures and hyperactivity. Recently, several studies identified altered GABAergic activity through chloride-permeable $GABA_A$ receptors as one of the main contributors to impaired cognitive performance in the Ts65Dn mouse model of DS. Data from adult Ts65Dn mice and DS individuals showed an increased expression of the chloride importer NKCC1. As a result, intracellular chloride concentration is higher in Ts65Dn mice and GABAergic responses are depolarizing (*vs* hyperpolarizing and inhibitory). Accordingly, treatment with the FDA-approved diuretic and NKCC1 inhibitor bumetanide during adulthood rescues inhibitory GABAergic transmission and cognitive deficits in DS mice, although the beneficial effect of the treatment is rapidly lost upon drug withdrawal. However, hyperactivity and susceptibility to seizures are not rescued by bumetanide treatment in adulthood. Here, we investigated the long-term effects of early-in-life genetic and pharmacological interventions targeting NKCC1 by neuron-specific AAV9-mediated NKCC1 knockdown and bumetanide treatment during the first 2 weeks of development, respectively. We found a rescue in long-term memory in two different memory tasks in adult Ts65Dn animals after both interventions. Additionally, early NKCC1 downregulation rescued short-term memory, susceptibility to seizures and hyperactivity phenotype in Ts65Dn mice in adulthood. Notably, both early-in-life genetic and pharmacological interventions rescued the increased GABA-mediated spiking events in acute brain slices from adult trisomic animals. Finally, since bumetanide treatment of human infants can lead to deafness, we assessed ototoxicity in adult WT and Ts65Dn mice treated early in development and found no significant deficits in acoustic startle-response. Our results suggest that time-specific interventions possibly impacting on the trajectories of the developing brain could rescue cognitive performance and deficits that are not rescued by treatment in adulthood, avoiding the adverse diuretic effects of the required chronic adult treatment with bumetanide.

Introduction

The role of GABA in brain development

The emergence and functional maturation of neuronal circuits during brain development involves three distinct yet partially overlapping phases: an early, innate activity-independent phase, a second, activity-dependent phase driven by spontaneous patterns of neuronal activity and finally an experience-dependent phase of neuronal activity driven by sensory experience (Figure 1). The first phase dictated by genetic cues and the second by spontaneous neuronal activity are essential for neuronal progenitor proliferation and differentiation. During these two phases newly born neurons mature morphologically and migrate to their final locations in the brain, establishing a first set of neuronal connections (Khazipov, Esclapez et al. 2001, Spitzer 2006). After the functional maturation of the sensory organs, the final stage begins, and neuronal activity driven by sensory experience from the external environment refines the initial coarse neuronal circuitry: a circuitry that undergoes many changes throughout life and it is never static (Feller 1999, Leighton and Lohmann 2016, Hadders-Algra 2018).

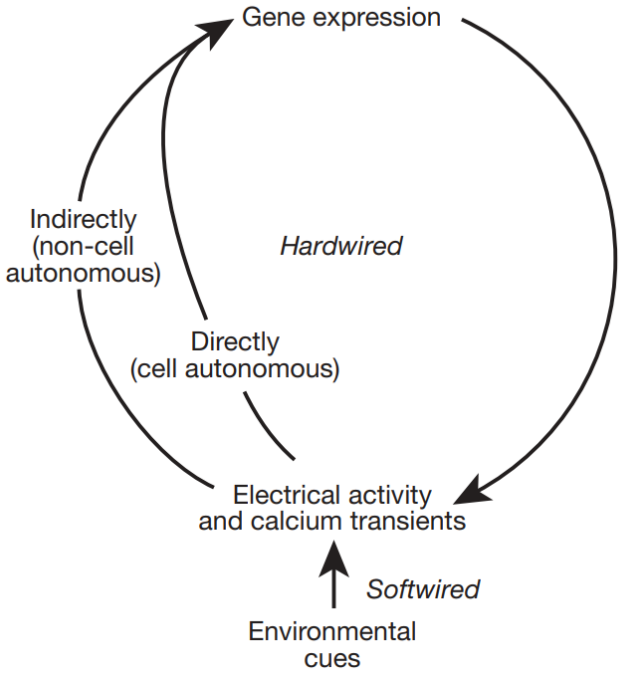


Figure 1. The interplay between gene expression, electrical activity and environment. At the first step of the hardwiring of early development genes coding for channels, receptors and ligands are expressed.

Ligand binding and gap junctions drive first electrical activity through transient elevations of calcium. This electrical activity in turn regulates gene expression both directly and indirectly. During this loop environmental cues impact electrical activity by softwiring. Adapted from (Spitzer 2006).

The first neurotransmitter to be functional in the developing neuronal networks is GABA (γ-aminobutyric acid) (Khazipov, Esclapez et al. 2001, Ben-Ari 2014). GABA is mostly known as the main inhibitory neurotransmitter in the adult CNS. However, during development, its action is mostly depolarizing and excitatory. GABA is synthetized in the CNS of vertebrate organisms from L-glutamic acid by the L-glutamic decarboxylase (GAD). GAD is present in two isoforms, GAD65 and GAD67 (Paoletti and Davison 1971, Buddhala, Hsu et al. 2009). GABA exerts its action by binding to two different families of receptors: the ionotropic $GABA_A$ receptors and the metabotropic $GABA_B$ receptors. The assembly of 5 different subunits out of a total of nineteen potential subunits (α1-6, β1-3, γ1-3, δ, ε, θ, π, ρ1-3) form heterogeneous $GABA_AR$ complexes, which are ligand-gated ion channels. Different subunit combinations confer different functional properties to $GABA_A$Rs'. These properties relate to ionic gating dynamics, cellular localization, and physiological functions (Koduvayur, Gussin et al. 2014, Has and Chebib 2018). $GABA_AR$ functions are mediated by Cl^- currents, as $GABA_A$Rs are mostly permeable to chloride (Cl^-). The combination of subunits comprising $GABA_A$Rs determines the kinetics of the Cl^- currents. Those can be a slow and tonic extra-synaptic current or a fast and phasic synaptic current. Tonic currents are generated when low concentrations of GABA escape the synaptic cleft and activate extra synaptic $GABA_A$Rs, while phasic currents occur within the synaptic cleft, when GABA released in the synaptic cleft activates $GABA_A$Rs on the postsynaptic membrane (Brickley, Cull-Candy et al. 1999, Farrant and Nusser 2005, Cellot and Cherubini 2013). Tonic (extrasynaptic) currents plays a major role in mediating network activity during early brain development (Brickley, Cull-Candy et al. 1999, Farrant and Nusser 2005, Kilb, Kirischuk et al. 2013). Finally, GABA also acts on metabotropic $GABA_B$ receptors that form heterodimers of two different subunits ($GABA_B1$ and $GABA_B2$) localized on both pre- and postsynaptic membranes and operate through G proteins mediating inhibition in the adult brain (Ben-Ari, Gaiarsa et al. 2007), while during development $GABA_B$ receptor, which lacks coupling to potassium channels, has been identified to promote neuronal migration and morphological maturation through non-hyperpolarizing signaling (Bony, Szczurkowska et al. 2013).

Based on its concentration gradient across the membrane and the neuronal membrane resting potential, chloride can flow through $GABA_A$ receptors in both directions. Thus, when there is a low chloride concentration inside the cell the direction of the flow is inward. This determines a hyperpolarizing/inhibitory currents, which physiologically occurs in the adult CNS due to the high expression of the Cl^- exporter KCC2 (Figure 2). (Kaila, Price et al. 2014). Instead, during brain development, there is a high chloride concentration inside the cell. This high intracellular Cl^- concentration is driven by the high expression of the Cl^- importer NKCC1. The activation of the $GABA_AR$ determines a chloride efflux. This in turn leads to membrane depolarization, activation of voltage-gated calcium channels and removal of the Mg^{2+} block from NMDA receptors and calcium influx into the cell, causing further membrane depolarization (Kaila, Price et al. 2014). Interestingly, GABA exerts a central role in both the early stages of neurodevelopment, characterized by the organization of the CNS structure, and later, in the activity-dependent phase when the emerging network start to generate pattern of activity (Represa and Ben-Ari 2005). In the early stages of development, GABA exerts a trophic role in the development of neuronal networks by causing, calcium influx in the cell, which is vital for the activation of second messengers (i.e. calcium sensitive kinase (PKC) and calcium/calmodulin-dependent protein kinase II, CamKII), that take part in cell migration, differentiation and synapse formation (Noctor, Flint et al. 2001, Ben-Ari 2002, Takayama and Inoue 2010, Succol, Fiumelli et al. 2012, Cellot and Cherubini 2013, Ben-Ari 2014). Later in development, when the coarse circuit structure is built, GABA is responsible for the generation of the first pattern of activity able to create, maintain or remodel the network connections. Due to its depolarizing activity, it is able to generate spontaneous network bursts, network action potentials of various intervals and durations (Aguado, Carmona et al. 2003). This spontaneous activity is a key component of the developing brain, because of its fundamental role in orchestrating various developmental processes, from neurite elongation to migration and maturation of the neural network (Aguado, Carmona et al. 2003).

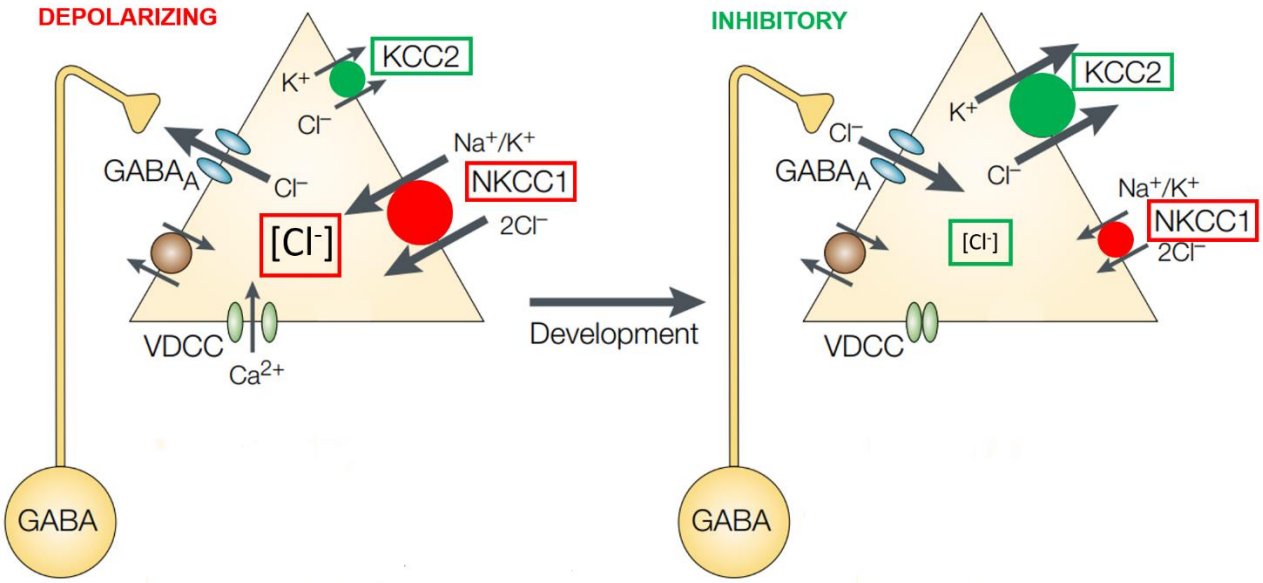


Figure 2. GABA exerts a depolarizing activity in immature neurons, while it has inhibitory action in adult. Left, High NKCC1 expression during development accumulates chloride inside the cells (30 mM), thus determining an outward direction of Cl^- upon $GABA_A$ receptor activation and depolarization of the membrane. Right, In adult neurons, the developmental upregulation of KCC2 expression determines a low Cl^- concentration (4-6 mM) inside the cell, thus an efflux of chloride and hyperpolarization of the membrane. Adapted from (Ben-Ari 2002).

These early patterns of spontaneous neuronal activity are evolutionary conserved during development and have been described in several species including the rodent, chick, ferret, rabbit and turtle (Wong 1999, Khazipov, Esclapez et al. 2001, Aguado, Carmona et al. 2003). Moreover, evidence from primate fetuses report early patterns of synchronized hippocampal network activity *in utero*. Those are similar to the giant depolarizing potentials (GDPs) described in the neonatal rodent hippocampus. However, they are shifted toward the fetal life in primates (Khazipov, Esclapez et al. 2001). Evidence about the role of depolarizing GABA during these early patterns of neuronal activity in humans has not been investigated yet. However, the presence of early patterns of synchronized activity has been described in human preterm babies by EEG and fMRI (Khazipov and Luhmann 2006, Tolonen, Palva et al. 2007, Arichi, Whitehead et al. 2017). The only data available in humans, derive from *in vitro* studies with neurons differentiated from human pluripotent stem cells (iPSCs). In these cells, early patterns of synchronous network activity is based mainly on gap junctions and emerges when the strong depolarizing GABA activity decreases

(Moore, Zhou et al. 2011, Makinen, Yla-Outinen et al. 2018). Recently, data in brain organoids reported the presence of GDP-like events mimicking the early network activity of the fetal brain (Zafeiriou, Bao et al. 2020). Interestingly, around differentiation day 40 the polarity of GABA switches from excitatory to inhibitory in the brain organoids, which coincides with reduced GDPs (Zafeiriou, Bao et al. 2020).

The switch in GABA polarity from depolarizing and mostly excitatory to hyperpolarizing and inhibitory coincides with the developmental upregulation of KCC2 and the disappearance of the network oscillations (Sernagor, Young et al. 2003, Leitch, Coaker et al. 2005). For at least some brain structures, that time coincides with the emergence of complex, region-specific behaviors (Dehorter, Vinay et al. 2012). Interestingly, this increase in KCC2 expression coincides with molecular changes in the composition of $GABA_AR$ subunits in the mature brain, which facilitates the refinement of inhibition (Kanold and Shatz 2006).

Finally, during the last phase of the development of the neural circuits, GABAergic transmission plays a fundamental role during the so-called critical periods (Figure 2). These are temporal windows of enhanced neuronal connectivity and plasticity driven by experience-dependent activity during brain development (Berardi, Pizzorusso et al. 2000, Hensch 2004, Hensch and Fagiolini 2005, Takesian and Hensch 2013, Begum and Sng 2017, Sommeijer, Ahmadlou et al. 2017, Hensch and Quinlan 2018, Zhang, Mu et al. 2018).

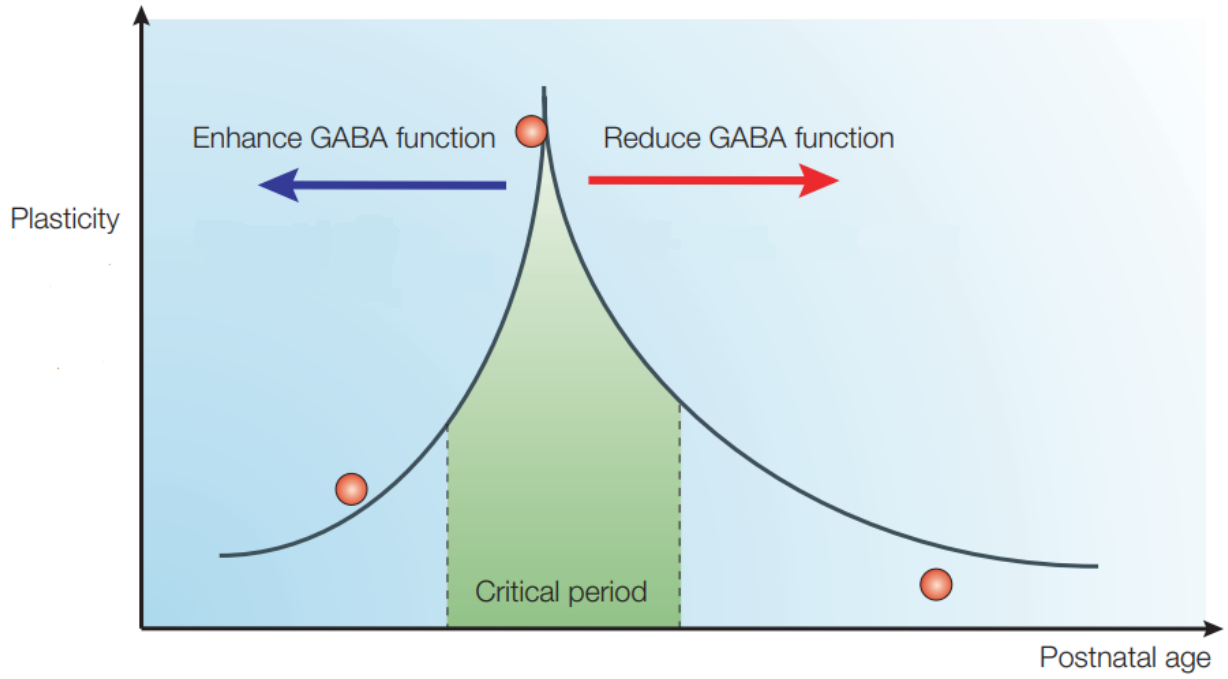


Figure 3. GABA regulates the opening and closure of critical periods. The onset and closure of the critical period for the most famous paradigm of monocular deprivation. The red circles indicate the onset, peak and end of the critical period. The blue arrow indicates that the onset of enhanced plasticity can be anticipated by enhancing GABA transmission, while the red arrow indicates that mediating reduction of GABA transmission prolongs the period of plasticity. Adapted from (Hensch 2005).

Physiological brain development and the role of chloride transporters

In recent years, the pivotal role that intracellular Cl^- concentration exerts in modulating GABAergic transmission and consequently the mechanisms orchestrating brain development has drawn attention for its implication in neurodevelopmental disorders. For the cation-chloride cotransporter family (CCCs), in neuronal cells, two cotransporters are the main regulators of Cl^- homeostasis: the sodium-potassium-chloride cotransporter isoform 1 (NKCC1) and the potassium-chloride symporter isoform 2 (KCC2) (Li, Tornberg et al. 2002, Blaesse, Airaksinen et al. 2009).

NKCC1 is highly expressed during development in immature neurons, where its inward-directed flow under physiological conditions keeps the intracellular chloride levels at high values (30mM). This leads to a depolarizing and mostly excitatory GABAergic activity during development (Dzhala, Talos et al. 2005, Achilles, Okabe et al. 2007). On the other hand, KCC2 is the only cotransporter from the CCC family which has solely neuronal expression. It is highly expressed in

mature neurons, where its outward-directed flow under physiological conditions keeps the intracellular chloride levels at low values (between 4-6 mM). This leads to hyperpolarizing and inhibitory GABA activity in the adult brain (Rivera, Voipio et al. 1999, Ben-Ari 2002, Stein, Hermans-Borgmeyer et al. 2004, Ben-Ari 2014, Delpire and Kahle 2017).

GABA switch in brain structures follows a caudal to rostral direction

Notably, the switch of GABAergic responses from depolarizing to hyperpolarizing during CNS development follows a caudal to rostral pattern, firstly occurring in evolutionarily older brain structures, which also develop firstly (e.g. spinal cord, brainstem), followed by higher structures (e.g. hypothalamus, thalamus and the neocortex) (Figure 4) (Watanabe and Fukuda 2015). Specifically, in the murine spinal cord, the mature pattern of low NKCC1 and high KCC2 expression can be observed as early as E15.5, promoting the switch of GABA polarity in motor neurons from depolarizing to hyperpolarizing at E17.5. Of note, this switch is abolished in KCC2 $^{-/-}$ motor neurons recorded at E18.5 confirming the important functional role of the two transporters (Hubner, Stein et al. 2001, Branchereau, Chapron et al. 2002, Delpy, Allain et al. 2008).

Moving caudally, in the developing hypothalamus, the presence of NKCC1 mRNA expression is not clear in the rodent embryos, and weak postnatally. However, the protein levels peak in the perinatal days (around E20-P0) and gradually decrease by P11 (Wang, Shimizu-Okabe et al. 2002, Perrot-Sinal, Sinal et al. 2007). Conversely, at E14.5, KCC2 mRNA is strongly present and maintains its expression into adulthood in rodents, while the protein is detectable within the first week of life in rodents (Li, Tornberg et al. 2002, Wang, Shimizu-Okabe et al. 2002, Perrot-Sinal, Sinal et al. 2007).

In the developing thalamus, NKCC1 mRNA is not present during embryonal life in rats but its expression increases and remains stable at postnatal ages (Wang, Shimizu-Okabe et al. 2002, Watanabe and Fukuda 2015). Conversely, KCC2 mRNA is already present in rodent dorsolateral nuclei of thalamus at the same time as the structure begins to form (E12) with the exception of the dorsomedial part, which expresses KCC2 later at E18 (Li, Tornberg et al. 2002, Wang, Shimizu-Okabe et al. 2002, Watanabe and Fukuda 2015).

In the developing cerebellum, NKCC1 mRNA is not detected in Purkinje cells, but it is detected only in granule cells at any postnatal age studied in rats. Specifically, NKCC1 mRNA is observed in the external germinal layer and in granule cells at P7 reaching adult levels by P21 (Mikawa, Wang et al. 2002). Conversely, KCC2 transcripts are found at E15.5 in mouse and at P1 in rat Purkinje cells, when the cells begin differentiation (Mikawa, Wang et al. 2002). Moreover, KCC2 mRNA is detectable already at P3 in mouse and at P7 in rat granule cells reaching adult levels by P21 similar to NKCC1 (Mikawa, Wang et al. 2002, Stein, Hermans-Borgmeyer et al. 2004).

In the developing hippocampus, NKCC1 transcripts are substantially present in hippocampal formation neuroepithelium at E18 and later at P1 in pyramidal cells layer of CA1 and CA3 regions in rats (Wang, Shimizu-Okabe et al. 2002), reaching the highest levels within the first week of life and then gradually decreasing by P15 (Plotkin, Snyder et al. 1997, Wang, Shimizu-Okabe et al. 2002, Pfeffer, Stein et al. 2009). Conversely, KCC2 mRNA transcripts are detected first in the CA3 region at E15.5 in mice. This is followed by transcript expression at the CA1 region at E18.5 and the dentate gyrus by the end of the first postnatal week, finally reaching adult levels by P15 (Stein, Hermans-Borgmeyer et al. 2004). In the same study, western blot analysis revealed considerable amounts of KCC2 protein perinatally in the mouse hippocampus (Figure 4) (Stein, Hermans-Borgmeyer et al. 2004).

In the developing neocortex, NKCC1 mRNA is detected in mice as early as E12.5 in the niches of neuronal progenitor cells in the neuroepithelium and in the ventricular zone of the ganglionic eminences, where its expression increases by E14.5 (Li, Tornberg et al. 2002, Watanabe and Fukuda 2015). In rats, strong levels of NKCC1 mRNA and protein are reported in differentiated cells of the cortical plate (Li, Tornberg et al. 2002, Watanabe and Fukuda 2015). NKCC1 protein expression reaches high expression levels in the postnatal neocortex between P3-P14 (Dzhala, Talos et al. 2005). Interestingly, KCC2 mRNA signals are not detectable in the developing neocortex in mice before birth (Figure 4) (Li, Tornberg et al. 2002, Wang, Shimizu-Okabe et al. 2002), while KCC2 protein levels are low during the first two postnatal weeks and they steadily increase by P21 (Dzhala, Talos et al. 2005).

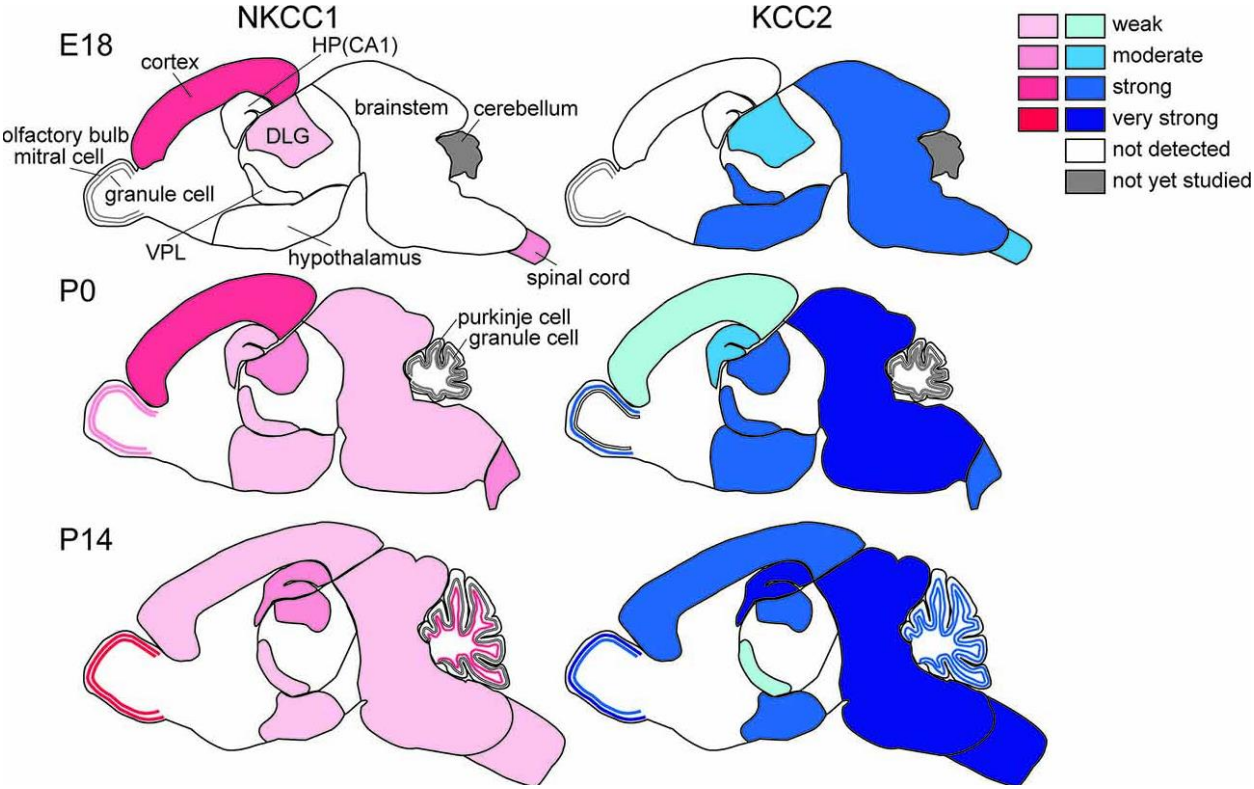


Figure 4. Developmental expression profiles of NKCC1 (left) and KCC2 (right) in the CNS. For the three time points studied (E18, P0, P14) and regions DLG: dorsal lateral geniculate nucleus; HP: hippocampus; VLP: ventral posterior thalamic nucleus. Colors indicate the level of expression of the two cotransports for NKCC1 (pink) and KCC2 (blue). Adapted from (Watanabe and Fukuda 2015).

Finally, in the epithelial tissue of the developing brain only NKCC1 is expressed, while KCC2 maintains its neuron-specific expression. Specifically, NKCC1 is highly expressed both in the developing and adult choroid plexus in rodents (Kanaka, Ohno et al. 2001, Li, Tornberg et al. 2002). Recent data suggest a key role for NKCC1 in water transport and the formation of the cerebrospinal fluid (CSF) in the adult brain, where it exhibits an unusual outward transport direction (Steffensen, Oernbo et al. 2018).

In the developing human brain (16 areas studied), studies report NKCC1 mRNA expression during the second trimester of gestation. NKCC1 increases with time and continues after birth (Hyde, Lipska et al. 2011, Sedmak, Jovanov-Milosevic et al. 2016). In particular, NKCC1 protein levels are high in the parietal cortex during the third trimester of gestation with a peak at post-conceptional week (PCW) 35 and gradually decrease from the first year of life to adulthood (Dzhala, Talos et al. 2005). Notably, KCC2-immunoreactivity is observed as early as PCW16 in a

subset of subplate neurons (Bayatti, Moss et al. 2008) and later PCW 25 in the hippocampus and entorhinal cortex (Dzhala, Talos et al. 2005, Sedmak, Jovanov-Milosevic et al. 2016). This is in line with KCC2 mRNA levels that are detected in prefrontal cortex and the hippocampus during the second trimester of gestation and gradually increase after birth (Hyde, Lipska et al. 2011), while KCC2 protein also increases over the first year of life (Dzhala, Talos et al. 2005). Interestingly, the overall ratio of NKCC1 to KCC2 is very high in pediatric human brains, gradually decreasing to finally reach stable adult levels by the second year of life (Jansen, Peugh et al. 2010).

The role of NKCC1 and KCC2 in the developmental processes

NKCC1 and KCC2 as the main chloride cotransporters determine GABAergic signaling and play a fundamental role in participating and regulating all the complex processes of the dynamic developing brain. Their role in cell proliferation, migration, synapse formation and maturation has been studied extensively in the literature.

NKCC1 regulates cell proliferation and apoptosis

In the developing brain, high NKCC1 mRNA expression has been reported in radial glial cells and the proliferative zones of the lateral and medial ganglionic eminences at E14.5 in *ex vivo* rat studies. Interestingly, NKCC1 was not expressed in post-mitotic migrating or differentiating neurons (Noctor, Flint et al. 2001, Li, Tornberg et al. 2002). This NKCC1-enriched expression in the proliferative zone of the subcortical region (but its absence in post-mitotic neurons) suggests a possible role in cell proliferation. This is supported by studies in NKCC1 knockdown mice that exhibit defects in the proliferation of neural precursor cells of the sub-ventricular zone (SVZ) and the neural progenitors of the lateral ganglionic eminences (Young, Taylor et al. 2012, Magalhaes and Rivera 2016). Interestingly, pharmacological blocking of NKCC1 by bumetanide had the same effect in inhibiting cell proliferation of neuronal precursors in the SVZ and rostral migratory stream (RMS) in mice (Figure 5) (Sun, Yu et al. 2012). Moreover, studies using bumetanide have reported the role of NKCC1 in the regulation of cell cycle progression in mouse oligodendrocyte

precursor cells *in vitro* (Fu, Tang et al. 2015). NKCC1 has also been demonstrated to play a role in apoptosis of Cajal-Retzius neurons, a population of neurons that mostly disappears early-in-life in the mouse developing cortex (Blanquie, Liebmann et al. 2017).

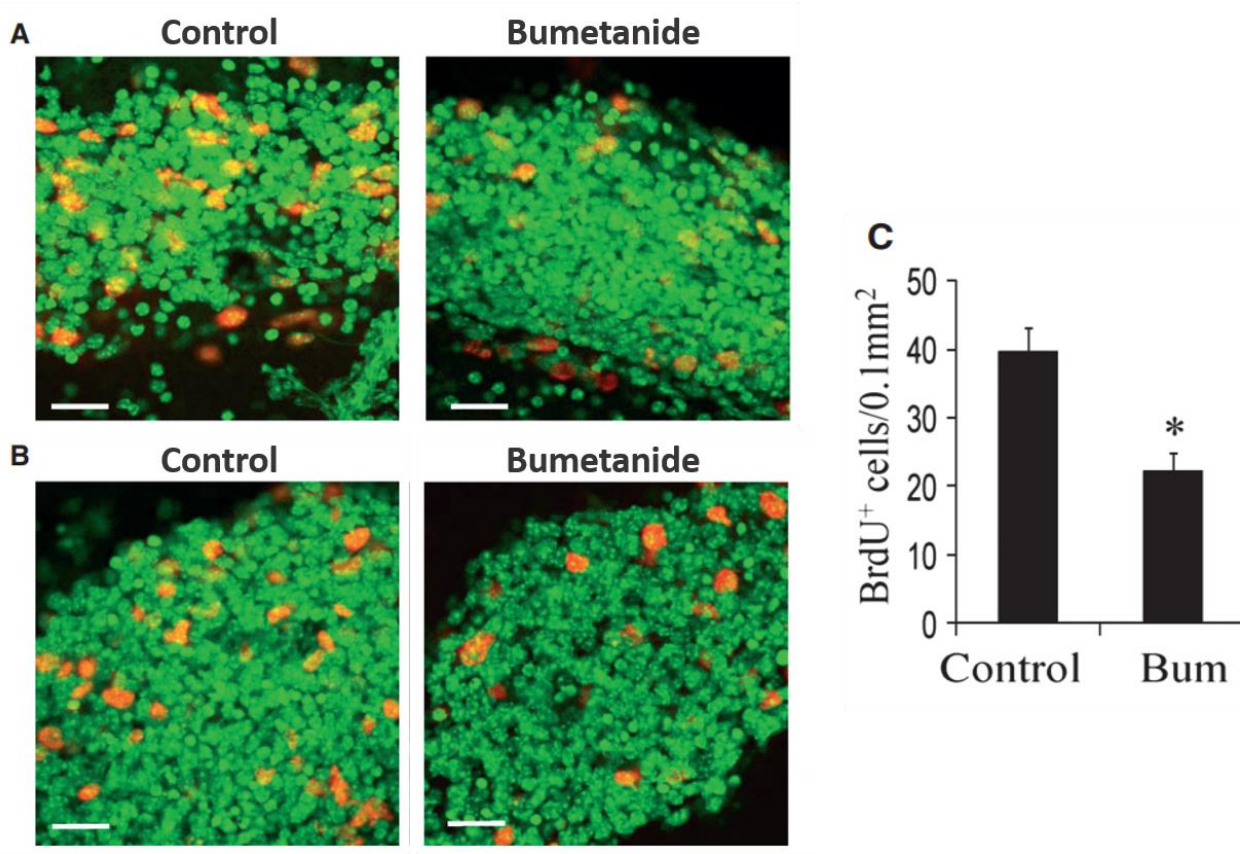


Figure 5. NKCC1 regulates proliferation in the SVZ and RMS precursors. Immunohistochemistry in slices of P18 mice for BrdU (red) and Hoechst staining for nuclei (green). **(A)** Precursors of the subventricular zone (SVZ) and **(B)** Precursors of the rostral migratory stream (RMS) in control condition or after incubation of bumetanide. **(C)** Quantification of BrdU-positive cells in control conditions and after bumetanide incubation. Adapted from (Sun, Yu et al. 2012).

Both NKCC1 and KCC2 play a role in neuronal migration

The depolarizing activity of GABAergic transmission in the developing neurons driven by the high expression of NKCC1 and intracellular chloride accumulation has been implicated in the migration of cortical neurons in the ventricular zone and the cortical plate in rats (Behar, Li et al. 1996, Behar, Schaffner et al. 1998, Shimizu-Okabe, Yokokura et al. 2002, Heck, Kilb et al. 2007). RNAi against NKCC1 and pharmacological blocking by bumetanide *in vitro* reduced migratory speed in

neuroblasts (Mejia-Gervacio, Murray et al. 2011). Indirect *in vivo* evidence supporting the role of NKCC1 in migration of newly-born neurons comes from a study, where authors demonstrate a disruption of cortical neuron morphology after NKCC1 knockdown by *in utero* electroporation in mice, without however investigating the migration of neurons (Wang and Kriegstein 2008). Notably, activation of $GABA_A$ receptor facilitated the migration of interneurons in the marginal zone (MZ) in an intracellular calcium-dependent manner. This process was slowed by bumetanide-driven NKCC1 inhibition (Inada, Watanabe et al. 2011), demonstrating the role of NKCC1 in tangential migration. Moreover, several lines of evidence suggest that increased NKCC1 to KCC2 ratio in cortical neurons in rodents underlies pathologies related to morphological abnormalities during brain development (such as micro-gyral cortical malformations and neuronal ectopy) (Shimizu-Okabe, Okabe et al. 2007, Koyama, Tao et al. 2012, Fukuda and Wang 2013). Interestingly, interventions targeting NKCC1 (RNAi-mediated NKCC1 knockdown and pharmacological inhibition by bumetanide) rescued aberrant migration of granule cells in the dentate gyrus (DG) in a rat model of febrile seizures (Koyama, Tao et al. 2012), and the tangential migration in an ethanol-induced interneuronopathy mouse model (Skorput, Lee et al. 2019).

During the late stages of migration, when migrating precursors settle down and begin to develop their neurites, expression of KCC2 increases leading to a subsequent reduction in the intracellular chloride concentration (roughly from 30 mM to 10mM) (Owens, Boyce et al. 1996, Yamada, Okabe et al. 2004, Achilles, Okabe et al. 2007). Several lines of evidence suggest that KCC2 mediates migration of interneurons at their final stage (Bortone and Polleux 2009), where its upregulation works as a stop signal both *in vitro* and *in vivo* in mice and in organotypic cultures from a ferret model of cortical dysplasia (Bortone and Polleux 2009, Miyoshi and Fishell 2011, Inamura, Kimura et al. 2012, Abbah and Juliano 2014). Interestingly, KCC2 expression was not found in the migratory interneurons but only after they settled in the cortical plate (Inada, Watanabe et al. 2011). This pattern of KCC2 expression has also been demonstrated in excitatory neurons in rats, where the cotransporter increases expression levels only after the neurons have completed their migration across different cortical layers (Cancedda, Fiumelli et al. 2007). Notably, overexpression of KCC2 in newly born excitatory cortical neurons by *in utero* electroporation did not affect their migration in rats (Cancedda, Fiumelli et al. 2007). This may possibly be because the depolarizing GABA signaling is preserved by taurine-mediated inhibition of KCC2, which is highly expressed at embryonic stages (Inoue, Furukawa et al. 2012). In line

with the results above, downregulation of KCC2 using the environmental toxin (BPA) and a KCC2 antagonist rescues the abnormal features of neuronal migration observed after increase in KCC2 levels in ferrets (Djankpa, Lischka et al. 2019). Recently, in a mouse model of intellectual disability related to loss-of-function of *OPHN1* (Oligophrenin 1), interneuron migration was found to be impaired with lower number of cells reaching their final position. The impaired migration was due to an altered Cl^- homeostasis driven by premature KCC2 upregulation, which was rescued by application of KCC2 blocker, VU0463271 (Maset, Galla et al. 2021).

NKCC1 and KCC2 have a role in neuronal maturation

Both NKCC1 and KCC2 play key roles in neuronal morphological maturation (neurite branching and maintenance, inhibitory and excitatory synaptogenesis and plasticity of synapses) (Nakanishi, Yamada et al. 2007, Wang and Kriegstein 2008, Pfeffer, Stein et al. 2009, Khalilov, Chazal et al. 2011, Kaila, Price et al. 2014, Sedmak, Jovanov-Milosevic et al. 2016). Specifically, NKCC1 knockdown by *in utero* electroporation in mouse excitatory cortical neurons alters the physiological morphological parameters of dendritic arbors, spine density and spine length of both excitatory and inhibitory synapses (Wang and Kriegstein 2008, Young, Taylor et al. 2012). However, Pfeffer et al., reported no significant morphological alterations of hippocampal arborizations in NKCC1 $^{-/-}$ mice. Although, they found a delay in the maturation of GABAergic and glutamatergic synapses (Pfeffer, Stein et al. 2009). Moreover, pharmacological inhibition of NKCC1 disrupted AMPA synapse maturation, indicating that GABA depolarization cooperates with NMDA receptor activation to regulate excitatory synapse formation during mouse cortical development (Wang and Kriegstein 2011). Interestingly, NKCC1 expression has been found in the growing neurites and genetic and pharmacological interference with NKCC1 led to abolished outgrowth of the developing neurites in PC12 cells (Nakajima, Miyazaki et al. 2007, Nakajima and Marunaka 2016). Interestingly, NKCC1 participate in neurite regeneration of injured adult rat neurons *in vivo* (Pieraut, Laurent-Matha et al. 2007, Modol, Santos et al. 2015).

KCC2 is expressed in the neuronal cell soma and dendrites, including dendritic spines. Increase of KCC2 expression correlates with synaptogenesis in the rat hippocampus (Gulyas, Sik et al. 2001). The role of KCC2 in synapse formation has been demonstrated in a series of studies. Premature

expression of KCC2 in a subpopulation of cortical progenitors by *in utero* electroporation in rats has a profound effect on morphological maturation, with decreased number and length of neurites (Figure 6) (Cancedda, Fiumelli et al. 2007) together with an increased number of dendritic spines (Fiumelli, Briner et al. 2013). The dual role of KCC2 in both dendritic growth and in spine maintenance could be the possible reason behind the seeming discrepancy after premature KCC2 upregulation. Specifically, the role of KCC2 in dendritic growth requires GABA depolarizing signaling. Conversely, the structural role of KCC2 in dendritic spine formation is Cl^--independent. Indeed, transfection with the N-terminal-deleted KCC2 (which has no transporter activity) rescued the deficits induced by premature KCC2 overexpression (Fiumelli, Briner et al. 2013). As a molecular mechanism of this spinogenesis KCC2 demonstrated to directly interact with the cytosolic actin-associated protein 4.1N (Li, Khirug et al. 2007). Studies on KCC2 and excitatory synapses show that dendritic spine heads (the location of AMPA and NMDA receptor) are KCC2-enriched (Chamma, Chevy et al. 2012, Blaesse and Schmidt 2015), and KCC2 has a role in constraining lateral diffusion of AMPA receptors, and in regulating their content at the spine (Gauvain, Chamma et al. 2011, Chevy, Heubl et al. 2015, Llano, Smirnov et al. 2015). In line with previous studies, KCC2 ($^{-/-}$) mice show profound alterations in synaptic and neuronal network activity in the CA3 hippocampal region (Khalilov, Chazal et al. 2011). Finally, premature KCC2 overexpression was also found in a rat model of atypical febrile seizure and reduction in its levels rescued the morphological alterations in spine density and the susceptibility to seizures phenotype (Awad, Sanon et al. 2016). In humans, a KCC2 variant has been found in an Australian family with febrile seizures with a reduction in the number of dendritic spine (Puskarjov, Seja et al. 2014).

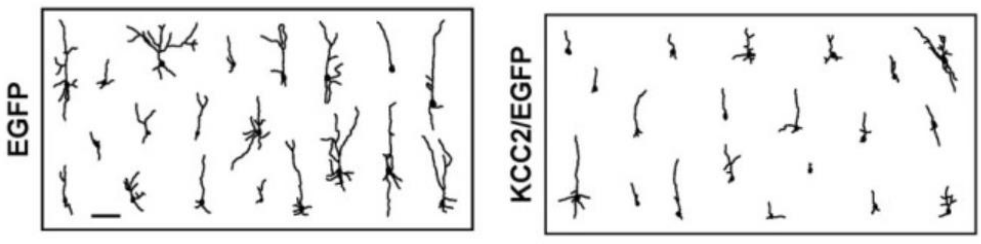


Figure 6. Premature KCC2 overexpression has a profound effect on morphological maturation. Two dimensional projection of dendrites of neurons in II/III layers of the cortex in rats (P6) electroporated *in utero* either with EGFP (left) or KCC2/EGFP (right). Adapted from (Cancedda, Fiumelli et al. 2007).

NKCC1 and KCC2 regulate critical periods in the developing brain

Brain plasticity early-in-life is much higher than later in adulthood. The critical period for sensory system plasticity has been widely investigated in the visual system, starting from the pioneering study of Wiesel and Hubel in 1965 with one of the most famous experiments in neuroscience. They showed that monocular deprivation in kittens leads to permanent alterations in the brain organization and permanent loss of vision. On the other hand, in adult cats there is no such an effect. This proved the existence of critical periods in which the developing brain is highly susceptible to changes induced by sensory experience (Wiesel and Hubel 1965).

In the case of the visual cortex, proper development of GABAergic transmission is crucial for both the opening and the closure of the critical period plasticity. Indeed, manipulation of inhibition, by prematurely enhancing or reducing GABAergic signaling during development controls the onset of the critical period plasticity in rodents (Huang, Kirkwood et al. 1999, Fagiolini and Hensch 2000, Iwai, Fagiolini et al. 2003, Hensch 2004, Hensch and Fagiolini 2005). Notably, reducing GABAergic activity in adult animals reopens the critical period in the visual cortex (Harauzov, Spolidoro et al. 2010). More recently, Deidda and colleagues demonstrated that depolarizing GABAergic signaling during early postnatal brain development plays a key role in the duration of the critical period for visual cortical plasticity. In particular, they reported that pharmacological inhibition of NKCC1 with bumetanide from P3 until P8 in rats extended the duration of the critical period into young adulthood (P35) in bumetanide-treated rats, with a mechanism dependent on the neurotrophin BDNF (Figure 7) (Deidda, Allegra et al. 2015).

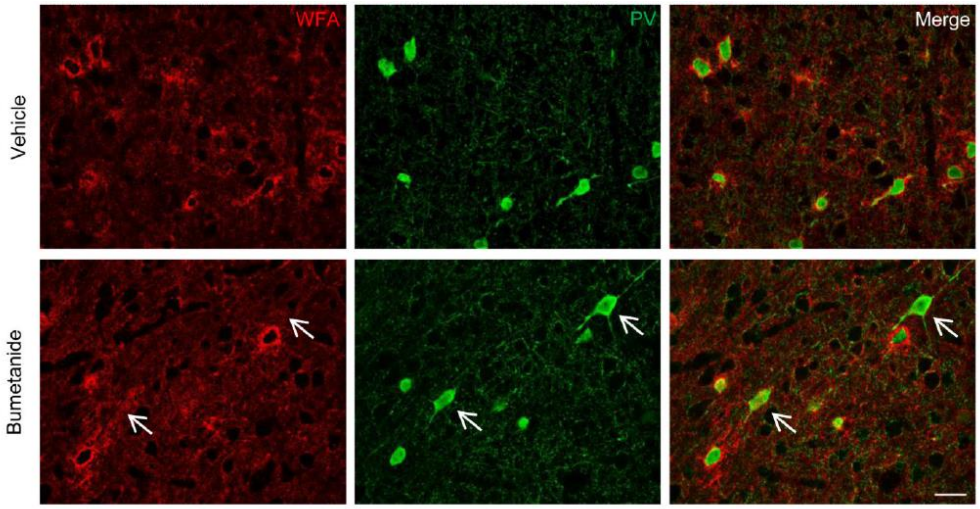


Figure 7. Early interference of depolarizing GABA prolongs critical period in the visual system by inducing changes in plasticity markers at P35. Visual cortex coronal sections from rats (P35) treated either with vehicle or bumetanide between P3-P8. Immunostaining for (from left to right) WFA-perineuronal nets (red), parvalbumin interneurons (PV) (green), and double-positive. Arrows indicate parvalbumin interneurons not surrounded by PNNs. Adapted from (Deidda, Allegra et al. 2015).

Expression and functional profiles of other CCC isoforms in the developing brain

The NKCC family apart from NKCC1 contains also NKCC2 isoform. Epithelial cells of the ascending limb of loop of Henle in the kidney and the macula densa express high levels of NKCC2 (Edwards, Castrop et al. 2014, Delpire and Gagnon 2018). There, NKCC2 isoform facilitates the reabsorption of sodium and chloride ions into the blood (Edwards, Castrop et al. 2014, Delpire and Gagnon 2018). Pharmacological agents blocking NKCC2 lead to excessive diuresis (including pronounced natriuresis and kaliuresis) (Becker, Nothwang et al. 2003, Gamba and Friedman 2009, Hannemann, Christie et al. 2009, Schiessl and Castrop 2015). Interestingly, loss-of-function mutation of *NKCC2* gene results in Bartter's syndrome, which is characterized by hypokalemic alkalosis, hyponatremia and hypotension (Simon, Karet et al. 1996). In humans, NKCC2 is strongly expressed in the epithelial layer of the endolymphatic sac of the inner ear (Kakigi, Nishimura et al. 2009). Recently, NKCC2 immunoreactivity was also reported in the brain in the posterior pituitary gland, specifically in the vasopressin and oxytocin-positive neurons in the hypothalamic-neurohypophyseal system in the rat brain (Konopacka, Qiu et al. 2015). In humans,

NKCC2 mRNA was not detected in any brain region studied (Sedmak, Jovanov-Milosevic et al. 2016).

Of the KCC family of Cl^- extruders, four isoforms have been reported so far, KCC1, KCC2, KCC3 and KCC4, and all of them are expressed in the developing brain (Li, Tornberg et al. 2002, Kaila, Price et al. 2014). KCC1 isoform is expressed in both neuronal and non-neuronal cells at relatively low levels, but it is highly enriched in epithelial cells, especially at the choroid plexus. There, KCC1 mRNA was exclusively detected during mouse brain development (Kanaka, Ohno et al. 2001, Li, Tornberg et al. 2002). KCC1 mRNA levels have also been reported in neurons and glial cells in several regions (hippocampus, olfactory bulb, posterior hypothalamic nucleus) of the adult rat brain (Kanaka, Ohno et al. 2001). Interestingly, KCC1 has been found to negatively regulate the NGF-dependent neurite outgrowth in vitro in PC12 cells (Nagao, Nakajima et al. 2012). Outside the CNS, KCC1 has been found in bone, testis, ovary, and kidney (Garneau, Marcoux et al. 2017). In humans, KCC1 mRNA was first observed in the cerebellar cortex at embryonic stages (PCW 10–13) and also found in several other brain regions (hippocampus, striatum, and thalamus) at PCW 21 and birth (Sedmak, Jovanov-Milosevic et al. 2016). Nevertheless, KCC1 expression is relatively low in the human cortex (Kaila, Price et al. 2014).

KCC3 isoform is widely expressed in the rodent CNS both in neurons and other cells throughout brain development, and its levels undergo a developmental increase (Pearson, Lu et al. 2001, Boettger, Hubner et al. 2002). Interestingly, two KCC3 isoforms show specific expression patterns, KCC3a is expressed in the brain, whereas KCC3b is restricted to tissues outside the CNS (Blaesse, Airaksinen et al. 2009). During brain development in rodents, KCC3 mRNA is weakly present during embryogenesis (Li, Tornberg et al. 2002), but both the mRNA and the protein are widely expressed in the adult brain (cortex, hippocampus, brainstem and cerebellar Purkinje neurons) (Pearson, Lu et al. 2001, Shekarabi, Salin-Cantegrel et al. 2011). Functionally, KCC3 has been implicated in myelination because of its expression in white matter-rich structures (spinal cord and peripheral nerves) (Pearson, Lu et al. 2001). Indeed, KCC3 $^{-/-}$ mice exhibit axonal swelling, hypomyelination, and demyelination in sciatic nerves (Howard, Mount et al. 2002, Byun and Delpire 2007). These deficits reported in rodents recapitulate most of the human peripheral neuropathy symptomatology (Bowerman, Salsac et al. 2017). In fact, loss-of-function mutations in *KCC3* gene have been associated with Andermann syndrome, a human peripheral neuropathy

associated with agenesis of the corpus callosum characterized by severe sensorimotor neuropathy, locomotor abnormalities and areflexia (Howard, Mount et al. 2002, Uyanik, Elcioglu et al. 2006, Bowerman, Salsac et al. 2017). More recently, KCC3 was reported to contribute to cell volume regulation in mouse peripheral nerve fibers (Flores, Schornak et al. 2019). Along with KCC1, KCC3 has been found to be expressed outside the CNS in bone, testis, ovary, and kidney (Garneau, Marcoux et al. 2017). In humans, KCC3 mRNA was described in the cortex, cerebellum, hippocampus, amygdala, striatum, and thalamus in all the stages of development and adulthood with peak of expression during prenatal period and a developmental decrease in expression (age range: 5 PCW–82 years (Sedmak, Jovanov-Milosevic et al. 2016).

KCC4 isoform undergoes developmental decrease in adult rodent brain. During embryonic mouse brain development, KCC4 is expressed in the choroid plexus, peripheral ganglia, and the nucleus of the trigeminal nerve and most abundantly in the ventricular zones (Li, Tornberg et al. 2002). In the adult CNS, KCC4 is present at low level in cranial nerves, brainstem and spinal cord (Karadsheh, Byun et al. 2004). The major phenotype of KCC4 ($^{-/-}$) mice is deafness suggesting that the KCC isoform plays a role in cochlear development, along with NKCC1. KCC4 loss-of-function leads to the cell death of hair cells possibly by osmotic perturbation or membrane depolarization (Boettger, Hubner et al. 2002). In humans, KCC4 mRNA was detected at low levels in the cortex both at prenatal and postnatal ages (Kaila, Price et al. 2014), but was not found in another study in any brain region studied (Sedmak, Jovanov-Milosevic et al. 2016).

Chloride transporter dysregulation in neurodevelopmental disorders

Neurodevelopmental disorders (NDs) are chronic psychiatric/neurological conditions that are present in up to 4–5% of the population (Mitchell, 2011). As already discussed above, the highly orchestrated brain developmental processes (proliferation, migration and cell fate specification) rely on an inextricable link between the genome and the environment (Esposito, Azhari et al. 2018). In the developing brain these processes are not merely driven by predetermined genetic programs, but they also rely on phenotypic checkpoints. These are, for example, functional feedbacks on time-specific and region-specific developmental processes driven by the integration of genetic and environmental cues in the assembly of neuronal networks (Ben-Ari and Spitzer

2010). The recent advances in genomic and functional genomic analysis has resulted in the identification of hundreds of mutations that possibly predispose to the development of NDs (O'Donovan and Owen 2016, Tarlungeanu and Novarino 2018). Notably, in the majority of NDs, a combination of genetic predisposition and environmental factors acting on the first years of life in humans has been suggested to be the trigger for the development of the disease (Kim and Leventhal 2015, Mitchell 2015). Moreover, epigenetic modifications (e.g. histone deacetylation, DNA chromatin methylation and non-coding RNAs) constitute a link between the environment and the genetic code and can reversibly regulate gene expression with implications for neurodevelopmental disorders (Wade, Gegonne et al. 1999). In particular, the epigenetic patterning during brain development is considered of high importance for the imprinting and gene dosage regulation, and can thus induce long-lasting changes from molecular to behavioral level (Feng, Fouse et al. 2007, Murgatroyd, Patchev et al. 2009, Nagy and Turecki 2012, Yoon, Vissers et al. 2018).

Any deviations from the typical development of the nervous system that leads to clinical symptoms is considered a neurodevelopment disorder (ND) by definition. Neurodevelopmental disorders include genetic syndromic diseases, such as Rett syndrome, Down syndrome and Fragile X, and also multifactorial disorders such as non-syndromic autism spectrum disorders, intellectual disability, ADHD and epilepsy among others (Harris 2014, Ghiani and Faundez 2017, Tarlungeanu and Novarino 2018, Cardoso, Lopes-Marques et al. 2019). Interestingly, also neuropsychiatric disorders traditionally considered belong to adults such as schizophrenia, bipolar disorder and anxiety disorders are now considered neurodevelopmental disorders, having their origins in the developing brain (Leonardo and Hen 2008, Owen and O'Donovan 2017, Fleiss, Rivkees et al. 2019). Although they display very different etiologies, the majority of NDs share common phenotypic features with mild to profound impairments in executive functions (learning deficits, memory, attention, emotional regulation, behavioral flexibility, sociability, and self-regulation) and some comorbidity (e.g., increased seizure susceptibility hyperactivity and sleep disorders, high levels of inflammation).

Interestingly, many of these common phenotypic features have been attributed to alterations found in GABAergic transmission. In particular, several studies demonstrated a high intracellular chloride accumulation in neurons and a depolarizing GABAergic activity, attributed to an altered

NKCC1/KCC2 cotransporter ratio in a wide range of NDs. Those include epilepsy, autism spectrum disorders, Down syndrome, Fragile X syndrome, Asperger syndrome, Rett syndrome, schizophrenia, Dravet syndrome, tuberous sclerosis complex and traumatic brain injury (Medina, Friedel et al. 2014, Jaggi, Kaur et al. 2015, Wu, Che et al. 2016, Ben-Ari 2017, Schulte, Wierenga et al. 2018).

The focus of both animal and clinical studies has been the restoration of physiological intracellular Cl^- concentration by genetic or pharmacological interventions aiming at regulating NKCC1 and KCC2 expression/function. This has been mostly done with the pharmacological inhibition of NKCC1 with bumetanide (Medina, Friedel et al. 2014, Jaggi, Kaur et al. 2015, Ben-Ari 2017, Schulte, Wierenga et al. 2018). Other studies investigated restoration of physiological Cl^- concentration by enhancing KCC2 transport, with the KCC2 activator CLP257 (Gagnon, Bergeron et al. 2013). However, the KCC2 selectivity of CLP257 has been debated as Cardarelli and colleagues argued that the mechanism of action is the potentiation of $GABA_A$ receptor activity and not KCC2 activation (Cardarelli, Jones et al. 2017). The hypothesis was rejected by Gagnon and colleagues by replicating their results, thus suggesting the need for further investigation (Gagnon, Bergeron et al. 2017). Finally, recent studies evaluate additional targets for restoration of the NKCC1/KCC2 expression/function for therapeutic purposes. In particular, the insulin-like growth factor-1 (IGF-1) reported to decrease the NKCC1/KCC2 ratio in the developing neurons in rats, facilitating in this way the developmental switch of GABA from depolarizing to hyperpolarizing (Baroncelli, Cenni et al. 2017). Moreover, the WNK-SPAK kinase is a potential therapeutic target regulating the two cotransporter ratio by activating NKCC1 and deactivating KCC2 (Kahle, Rinehart et al. 2010, de Los Heros, Alessi et al. 2014, Kahle, Khanna et al. 2015).

Epilepsy

Epilepsy is a neurological disorder characterized by the predisposition to generate spontaneous recurrent seizures with or without motor manifestation, and varying degrees of impaired awareness. Epileptic seizures are caused by excessive or hypersynchronous neuronal activity in the brain with focal or generalized onset. (Chang, Lowenstein et al. 2003, Falco-Walter, Scheffer et al. 2018). Evidence from studies both in rodent models of epilepsy and humans report CCC

dysfunction, suggesting that neuronal hyperexcitability and hypersynchronous neuronal activity is the result of an alteration of the balance between excitation to inhibition (E/I) synaptic activity (Woo, Lu et al. 2002, Huberfeld, Wittner et al. 2007). Interestingly, an imbalance in NKCC1 and KCC2 activity leading to depolarizing GABAergic activity has been observed in several animal models of epilepsy (Ben-Ari 2017, Di Cristo, Awad et al. 2018).

The first studies investigating therapeutic interventions targeting restoration of physiological Cl^- homeostasis found that inhibition of NKCC1 with either furosemide or bumetanide caused a cease in the epileptic activity both *in vitro* and *in vivo* in rats (Hochman, Baraban et al. 1995, Schwartzkroin, Baraban et al. 1998, Hochman, D'Ambrosio et al. 1999). Notably, bumetanide treatment rescued the increased susceptibility to seizures in the developing brain facilitated by the high NKCC1 expression both *in vitro* and *in vivo* in neonatal rats (Dzhala, Talos et al. 2005). Moreover, in the kindling-induced seizure model for rodents, NKCC1 was found to be increased in the amygdala in rats (Okabe, Ohno et al. 2002), and KCC2 downregulated in the hippocampi of mice (Rivera, Li et al. 2002). In line with the above studies, KCC2-deficient mice exhibit increased frequency of seizures (Woo, Lu et al. 2002). More recent works in rodent models of epilepsy confirm the role of an altered NKCC1/KCC2 ratio in the pathogenesis of epilepsy, with the use of bumetanide to be the most commonly used intervention with positive outcomes in a series of preclinical studies (Dzhala, Brumback et al. 2008, Li, Zhou et al. 2008, Mazarati, Shin et al. 2009, Nardou, Ben-Ari et al. 2009, Dzhala, Kuchibhotla et al. 2010, Edwards, Shah et al. 2010, Almeida, Scorza et al. 2011, Koyama, Tao et al. 2012, Cleary, Sun et al. 2013, Loscher, Puskarjov et al. 2013, Reid, Riazi et al. 2013, Eftekhari, Mehrabi et al. 2014, MacKenzie and Maguire 2015, Marguet, Le-Schulte et al. 2015, Robel, Buckingham et al. 2015, Tollner, Brandt et al. 2015, Baek, Yi et al. 2016, MacKenzie, O'Toole et al. 2016, Sivakumaran and Maguire 2016, Zhang, Xu et al. 2016, Hu, Yang et al. 2017, Santos, Rodrigues et al. 2017, Wang, Wang et al. 2017, Amadeo, Coatti et al. 2018, Kelley, Cardarelli et al. 2018, Kharod, Carter et al. 2018). Finally, in a mouse model of maternal immune activation during gestation resulted in changes of KCC2 levels in offspring, mediated by increased binding of the repressor factor RE1 subsequently leading to transcriptional downregulation of KCC2 and increasing the susceptibility to epileptic seizures (Corradini, Focchi et al. 2018). This evidence support the hypothesis that early-in-life environmental insults can also induce changes in GABAergic system leading to epilepsy.

Interestingly, evidence from human studies show an imbalance in the NKCC1/KCC2 ratio, with NKCC1 upregulation and/or KCC2 downregulation present in epilepsy patients. Upregulation of NKCC1 was found in hippocampus and subiculum of temporal lobe epilepsy and focal cortical dysplasia patients (Palma, Amici et al. 2006, Huberfeld, Wittner et al. 2007, Munoz, Mendez et al. 2007, Sen, Martinian et al. 2007, Huberfeld, Blauwblomme et al. 2015). Altered expression of the NKCC1/KCC2 ratio has been reported also in cortical samples from epileptic children (Jansen, Peugh et al. 2010) and in rare forms of epilepsy (Aronica, Boer et al. 2007, Sen, Martinian et al. 2007, Kim, Fenoglio et al. 2008, Conti, Palma et al. 2011, Shimizu-Okabe, Tanaka et al. 2011, Pallud, Le Van Quyen et al. 2014). Recently, increased NKCC1 and decreased KCC2 has been observed in human brain samples of people with Dravet syndrome, an infantile encephalopathy characterized by severe epilepsy and cognitive impairment (Ruffolo, Cifelli et al. 2018). Bumetanide has been used as a treatment in clinical studies to treat epilepsy, ameliorating seizure frequency in temporal lobe epileptic patients and in a child affected by intractable multifocal seizures (Kahle, Barnett et al. 2009, Eftekhari, Mehrabi et al. 2014). However, the NEMO clinical trial, investigating the efficacy and safety of the use of bumetanide for the treatment of acute neonatal encephalopathy seizures (Pressler, Boylan et al. 2015), was interrupted due to poor bumetanide antiepileptic action and ototoxic effect in 3 out of 11 treated subjects (Ben-Ari, Damier et al. 2016). More recently, a pilot clinical trial with bumetanide to treat neonatal seizures reported reduction in seizure burden attributable to bumetanide over phenobarbital without increased serious adverse effects (Soul, Bergin et al. 2020). Nevertheless, this positive correlation between pathogenesis of epilepsy and decreased KCC2 levels has raised controversy as some other groups showed increased KCC2 expression in epileptic brain tissue from both rodent models (Galanopoulou 2008, Khirug, Ahmad et al. 2010, Awad, Sanon et al. 2016) and humans (Jansen, Peugh et al. 2010, Karlocai, Wittner et al. 2016). The upregulation or downregulation of KCC2 expression in epilepsy could be explained by a difference in the brain region studied, stage of disease, sex, or the influence of seizures themselves (Di Cristo, Awad et al. 2018). Indeed, KCC2 as an extruder causes potassium elevation in the extracellular environment, which could contribute to the lowering of the threshold for the generation of the seizures and to the synchronization of the epileptiform discharges (Di Cristo, Awad et al. 2018).

Autism spectrum disorders

Autism spectrum disorder (ASD) is a group of syndromes characterized by different etiologies but common core symptoms (i.e., repetitive behaviors, communication deficits in social interaction and language) (Pizzarelli and Cherubini 2011). Up to date, several risk factors for the development of ADS have been identified. However the specific etiology of ADSs is still poorly understood. The common core symptomatology suggests that some mechanisms driving ASD might be shared among different syndromes of the spectrum (e.g., Rett syndrome, Fragile X syndrome) (Percy 2011, Moss, Richards et al. 2013, Kaufmann, Kidd et al. 2017). Moreover, ASD, as most neurodevelopmental disorders, share high comorbidity with other developmental syndromes such as increased susceptibility to epileptic seizures (Lewis, Kesler et al. 2018).

ASD-related deficits develop often in utero or early-in-life (Stoner, Chow et al. 2014), a period when GABAergic signaling exerts a trophic role regulating the developmental processes. Interestingly, early evidence of altered GABAergic transmission underling ASD pathology, was reported when describing a paradoxical effect upon activation of $GABA_A$ with the benzodiazepine diazepam in autistic children (Marrosu, Marrosu et al. 1987). In particular, the authors reported anxiety and aggression in diazepam-treated autistic children, suggesting the presence of depolarizing GABAergic signaling in ASD (Marrosu, Marrosu et al. 1987). Following this evidence, preclinical studies in rodent models of ASD using valproic acid (VPA) treatment (in utero exposure to VPA is a risk factor for development of autism) reported downregulation of KCC2 and ASD-related behavioral deficits (Eftekhari, Mehrabi et al. 2014, Tyzio, Nardou et al. 2014). Notably, KCC2 heterozygous knockout mice exhibit alterations in social behavior (Anacker, Moran et al. 2019). Interestingly, bumetanide administration in VPA-treated pregnant rats resulted in the rescue of ASD-related behavioral deficits in their offspring (Eftekhari, Mehrabi et al. 2014, Tyzio, Nardou et al. 2014). More recently, a preclinical study targeting NKCC1, with treatment of bumetanide or a novel NKCC1-selective inhibitor, both rescued the social deficits and repetitive behaviors in young adult autistic (VPA) mice (Savardi, Borgogno et al. 2020).

Altered GABAergic transmission has also been identified as an underlying cause of ASD pathology in humans (Cellot and Cherubini 2013). Notably, a line of evidence in autistic patients reported that bumetanide treatment ameliorated the core symptoms of ASD, as assessed by the Childhood Autism Rating Scale (CARS). This suggests that bumetanide and interventions

targeting NKCC1 are promising for the treatment of ASD (Lemonnier and Ben-Ari 2010, Lemonnier, Degrez et al. 2012, Lemonnier, Villeneuve et al. 2017, Hadjikhani, Asberg Johnels et al. 2018). Moreover, bumetanide treatment of a young girl with Asperger syndrome, an autistic syndrome belonging to ASD, resulted in improvements of sensory deficits (Grandgeorge, Lemonnier et al. 2014). Notably, two Phase III clinical trials are ongoing investigating the safety and efficacy of oral Bumetanide treatment in children (2-7 years old) and adolescents (7-18 years old) with autism (NCT03715153 and NCT03715166 respectively).

Rett syndrome

Rett syndrome (RTT) is a neurodevelopmental disorder caused by intrauterine monogenic mutations in the X-linked Methyl-CpG-binding protein (MECP2) gene with a prevalence of 1 female in 10,000–15,000 female live births (Ip, Mellios et al. 2018). Mecp2 is a transcriptional repressor that binds to methylated DNA and regulates the transcription of a large number of genes (Chahrour and Zoghbi 2007). Rett syndrome individuals develop typically until the age of 6–18 months. However they then regress, exhibiting various symptoms (e.g., cognitive impairment, seizures, motor dysfunction, ASD-like symptoms and stereotypy among others (Ehinger, Matagne et al. 2018, Ip, Mellios et al. 2018). MeCP2 has been linked to GABAergic function, since its expression is approximately 50% higher in cortical GABAergic neurons compared to other types of neurons (Chao, Chen et al. 2010). Interestingly, in a MeCP2 mutant mouse model KCC2 was found reduced in the cortex (Banerjee, Rikhye et al. 2016). More recently, Lozovaya et al., showed that the developmental GABAergic shift is abolished at birth in CA3 pyramidal neurons of MeCP2 mutant mice, and that two weeks later GABA exerts strong excitatory activity that was restored by maternal bumetanide treatment one day before delivery (Lozovaya, Nardou et al. 2019). Interestingly, Tang and colleagues identified small-molecule compounds (KEECs) that increase KCC2 protein expression levels by acting on different signaling pathways (activation of the TRPV1 or SIRT1 pathways or inhibition of the FLT3 or GSK3β). Administration of KEECs rescued electrophysiological and morphological abnormalities of RTT neurons and ameliorated behavioral phenotypes related to respiration and locomotion in Mecp2 mutant mice (Tang, Drotar et al. 2019).

Evidence indicating a possible alteration in the GABAergic signaling in RTT humans showed an imbalance of NKCC1/KCC2 with a reduction of KCC2 in the cerebrospinal fluid (CSF) obtained from RTT patients (Duarte, Armstrong et al. 2013). This has been supported by deficits found in KCC2 expression in human RTT patient stem cell-derived neurons (Tang, Kim et al. 2016). Interestingly, insulin-like growth factor-1 (IGF1) treatment has been a therapeutic strategy to ameliorate the severity of the syndrome both in RTT mouse models (Tropea, Giacometti et al. 2009, Castro, Garcia et al. 2014, Banerjee, Rikhye et al. 2016) and in RTT patients (Khwaja, Ho et al. 2014, Pini, Congiu et al. 2016).

Fragile X syndrome

Fragile X syndrome (FXS) is a genetic disorder caused by intrauterine mutations in the X-linked FMR1 gene, which encodes for Fragile X mental retardation protein (FMRP). FMRP is a RNA-binding protein that acts as regulator of the translation of several mRNAs. FXS is the leading cause of inherited intellectual disability and a significant genetic contributor to ASD (Hodges, Reynolds et al. 2019). The clinical manifestation of FXS includes cognitive deficits, autism-like behavior, hypersensitivity to sensory stimuli and comorbidity with epilepsy, which is relatively benign and often resolved beyond childhood (Hagerman and Stafstrom 2009, Morel, Peyroux et al. 2018). Altered GABAergic signaling has been implicated in the pathophysiology of FXS. For example, in FXS KO mice the developmental switch in GABA polarity from depolarizing to hyperpolarizing is delayed, due to increased expression of NKCC1 (He, Nomura et al. 2014). Interestingly, Tyzio and colleagues found KCC2 to be decreased in hippocampal slices from FXS mice at P15 and P30 leading to elevated chloride levels. Maternal treatment with bumetanide one day before birth rescued the elevated excitability in CA3 neurons and the autism-like behavioral deficits in the *Fmr1* KO mice (Tyzio, Nardou et al. 2014). More recently, bumetanide-treated FXS mice during the critical period of somatosensory cortex plasticity rescued GABAergic signaling and synaptic plasticity and caused a long-lasting rescue of somatosensory circuit function (He, Arroyo et al. 2019). Conversely, Zeidler and colleagues found that treating FXS mice with bumetanide was insufficient to completely restore social impairment in the automated tube test. For that reason, they suggested a combination therapy of genetic reduction of mGluR5 expression along with bumetanide treatment, which in fact worsened social impairment (Zeidler, de Boer et al. 2017). In

humans, Lemonnier and colleagues treated a FXS child with bumetanide (Lemonnier, Robin et al. 2013). Interestingly, bumetanide administration resulted in the amelioration all the 5 clinical tests performed to evaluate autistic core symptoms (Lemonnier, Robin et al. 2013). Additionally, two Phase III double-blind randomized clinical trials (childhood-adolescence and adolescence-adulthood) targeting GABA signaling using arbaclofen, a GABAB agonists in autistic people (Berry-Kravis, Hagerman et al. 2017) did not succeed to demonstrate conclusive results on the efficacy of the treatment (Berry-Kravis, Lindemann et al. 2018).

Schizophrenia

Schizophrenia is a neurodevelopmental disorder, even if it is clinically diagnosed mostly in young adulthood. It is characterized by psychosis and a broad spectrum of behavioral, cognitive and social deficits, leading to disability and premature mortality (Lewis 2012, Marin 2016). The clinical symptomatology of schizophrenia can be divided into three categories: positive symptoms (e.g., hallucinations), negative symptoms (e.g., depression, avolition and apathy), and cognitive symptoms (Lewis 2012, Correll and Schooler 2020). The etiology of schizophrenia is yet mostly unknown, but it is considered a multifactorial disorder with both genetic and environmental contributors. Interestingly, changes in GABAergic neurotransmission have been reported in both rodent models and schizophrenic people (Gonzalez-Burgos and Lewis 2008, Hashimoto, Arion et al. 2008, Balu and Coyle 2011, Yang, Huang et al. 2015, Larimore, Zlatic et al. 2017). In particular, an altered NKCC1/KCC2 ratio was described in two different mouse models of schizophrenia (Yang, Huang et al. 2015, Larimore, Zlatic et al. 2017). Moreover, KCC2 mRNA expression was found to be significantly decreased in the hippocampus of schizophrenic people in comparison to controls, suggesting impaired Cl^- homeostasis due to increased NKCC1/KCC2 ratio (Hyde, Lipska et al. 2011). Furthermore, mutations in SLC12A2 and SLC12A5 genes, encoding for NKCC1 and KCC2, respectively, have been reported to increase the risk for developing schizophrenia in humans (Potkin, Turner et al. 2009, Merner, Chandler et al. 2015, Merner, Mercado et al. 2016). Two kinases (OXSR1 and WNK3) that regulate NKCC1 and KCC2 activity was found to be increased in the prefrontal cortex in patients suggesting a shift in the balance of chloride that can alter GABAergic signaling (Arion and Lewis 2011). Of, note, *in vitro* data from DiGeorge Syndrome (a condition that is highly comorbid to schizophrenia), NKCC1/KCC2 imbalance

caused hyperexcitability of the network which was rescued by bumetanide (Amin, Marinaro et al. 2017).

In agreement with the NKCC1/KCC2 imbalance found in human studies, treatment with bumetanide reduced the severity of the symptoms and hallucinations in schizophrenic people (Lemonnier, Lazartigues et al. 2016, Rahmanzadeh, Eftekhari et al. 2017), without recovering the total score of the general positive and negative syndrome scale (PANSS) and the brief psychiatric rating scale (BPRS) (Rahmanzadeh, Shahbazi et al. 2017). Oxytocin (intranasal administration) has been extensively used in studies to reduce the severity of symptoms of schizophrenia (Feifel, Macdonald et al. 2010, Goldman, Gomes et al. 2011, Pedersen, Gibson et al. 2011, Feifel, Macdonald et al. 2012, Davis, Lee et al. 2013, Fischer-Shofty, Brune et al. 2013, Lee, Wehring et al. 2013, Modabbernia, Rezaei et al. 2013, Davis, Green et al. 2014, Gibson, Penn et al. 2014, Woolley, Chuang et al. 2014, Shin, Park et al. 2015, Brambilla, Cotelli et al. 2016, Woolley, Chuang et al. 2017, Ota, Yoshida et al. 2018, Halverson, Jarskog et al. 2019). Nevertheless, several other studies suggest no effect of oxytocin on severity of schizophrenia symptoms (Horta de Macedo, Zuardi et al. 2014, Cacciotti-Saija, Langdon et al. 2015, Dagani, Sisti et al. 2016, Jarskog, Pedersen et al. 2017). Considering the ability of oxytocin to regulate GABA signaling during labor and birth, further investigation is needed to understand the mechanism of oxytocin for its therapeutic effect and if it is related to chloride transporter expression/function (Tyzio, Cossart et al. 2006, Khazipov, Tyzio et al. 2008, Eftekhari, Shahrokhi et al. 2014, Tyzio, Nardou et al. 2014, Leonzino, Busnelli et al. 2016, Ben-Ari 2018).

Tuberous sclerosis complex

Tuberous sclerosis complex (TSC) is a multiorgan, autosomal dominant genetic disorder resulting from loss-of-function mutations in the TSC1 or TSC2 genes. TSC is characterized by structural brain abnormalities such as dysplastic lesions (van Slegtenhorst, de Hoogt et al. 1997, Crino, Nathanson et al. 2006). Moreover, the clinical manifestation of TSC includes profound neurological impairments and often presents early-in-life with focal epilepsy, autistic behaviors, intellectual disability and general developmental delay (Crino, Nathanson et al. 2006, Ess 2006). Interestingly, an increased NKCC1/KCC2 ratio was found in TSC extracts from cortical tubers,

suggesting a possible involvement of altered GABAergic signaling in the pathogenesis of TSC (Talos, Sun et al. 2012, Ruffolo, Iyer et al. 2016). Notably, an altered GABA reversal potential was described in Xenopus oocytes grafted with cortical tissue membranes from TSC people (Ruffolo, Iyer et al. 2016).

Down syndrome

Down syndrome (DS), is the most common chromosomal disorder and the leading genetic cause of intellectual disability and congenital birth defects. It is caused by the triplication of a full or part copy of human chromosome 21 (Hsa21) (Nadel 2003, Antonarakis and Epstein 2006, Parker, Mai et al. 2010). DS occurs in approximately 1 of 800 live births (Bull 2020). The majority of cases (96 %) are caused by meiotic nondisjunction occurring in the egg, while less often it is caused by chromosomal translocation (3-4 %) or mosaicism (1-2 %) (Bull 2020). DS people exhibit developmental defects and growth delay and phenotypic features affecting most systems (Nadel 2003, Antonarakis and Epstein 2006, Antonarakis, Skotko et al. 2020). Individuals with DS are at high risk at developing multiple chronic conditions over their lifetime (i.e. congenital heart disease, hearing loss, overweight-obesity, sleep apnea) (Capone, Chicoine et al. 2018, Antonarakis, Skotko et al. 2020). Notably, almost all DS individuals manifest cognitive deficits to varying severity levels (Pennington, Moon et al. 2003, Dierssen 2012, Vicari, Pontillo et al. 2013, Grieco, Pulsifer et al. 2015).

There are several mouse models recapitulating aspects of the human trisomy. Although they all have limitations, they have transformed basic research in DS (Aziz, Guedj et al. 2018, Antonarakis, Skotko et al. 2020). The Ts65Dn mouse is the best characterized and most widely used animal model for DS (Reeves, Irving et al. 1995). Ts65Dn mice are characterized by a partial trisomy of *Mus musculus* chromosome 16 (MMU16), which is syntenic to the long arm of human chromosome 21, fused to the centromere of the murine chromosome 17 (Davisson, Schmidt et al. 1990, Antonarakis, Lyle et al. 2004). Interestingly, Ts65Dn mice recapitulate many features of DS phenotype, showing impairment in neuronal development (Belichenko, Masliah et al. 2004, Chakrabarti, Galdzicki et al. 2007, Contestabile, Fila et al. 2007, Chakrabarti, Best et al. 2010, Contestabile, Benfenati et al. 2010), defects of synaptic plasticity (Siarey, Stoll et al. 1997, Siarey,

Carlson et al. 1999, Kleschevnikov, Belichenko et al. 2004, Costa and Grybko 2005, Hanson, Blank et al. 2007, Contestabile, Greco et al. 2013), impaired hippocampus-dependent memory functions (Reeves, Irving et al. 1995, Holtzman, Santucci et al. 1996, Fernandez, Morishita et al. 2007, Contestabile, Greco et al. 2013), hyperactivity (Escorihuela, Fernandez-Teruel et al. 1995, Reeves, Irving et al. 1995, Sago, Carlson et al. 2000) increased susceptibility to seizures (Westmark, Westmark et al. 2010, Deidda, Parrini et al. 2015) and sleep disorders (Stewart, Persinger et al. 2007, Colas, Valletta et al. 2008, Das, Medina et al. 2015).

GABAergic transmission in Down syndrome

A first main finding suggesting an altered GABAergic transmission in trisomic models was the presence of an imbalance between excitatory and inhibitory inputs in the hippocampus of Ts65Dn mouse (Hanson, Blank et al. 2007). The same year, treatment with $GABA_A$ antagonists was shown to recover cognitive impairments in the same mouse model (Fernandez, Morishita et al. 2007). Moreover, agonist of GABAA α5 can restore LTP and attenuate cognitive performance (Braudeau, Delatour et al. 2011). Interestingly, the alteration in GABAergic activity of DS mice is also supported by studies reporting an increased number of GABAergic interneurons in the cortex and hippocampus of adult and adolescent Ts65Dn mice (Chakrabarti, Best et al. 2010, Perez-Cremades, Hernandez et al. 2010, Hernandez-Gonzalez, Ballestin et al. 2015). Interestingly, more recent in DS brain organoids and chimeric mouse brains studies have confirmed the increased interneuron production, due to upregulation of transcriptional regulators of interneurons mediated by increased OLIG2 expression (Xu, Brawner et al. 2019). Furthermore, an increase in spontaneous GABAergic postsynaptic events in CA1 pyramidal neurons was found in adult Ts65Dn mice. Nevertheless, no alterations were found in the frequency of mIPSCs or the probability of GABA release at the synapses (Chakrabarti, Best et al. 2010, Best, Cramer et al. 2012). Interestingly, further investigations revealed no alterations in the density GABAergic terminals and synapses of the CA1 region in adult Ts65Dn mice, but only on their distribution (Kurt, Davies et al. 2000, Belichenko, Masliah et al. 2004, Kurt, Kafa et al. 2004, Belichenko, Kleschevnikov et al. 2009). Conversely, Mitra and colleagues found increased frequency of mIPSC in the CA1 region of 2 weeks old Ts65Dn mice (Mitra, Blank et al. 2012), whereas other studies reported decrease of spontaneous inhibitory currents in the CA3 region of Ts65Dn mice

(Hanson, Blank et al. 2007, Stagni, Magistretti et al. 2013). Moreover, in the adult Ts65Dn dentate gyrus (DG) mIPSC were found to be increased (Kleschevnikov, Belichenko et al. 2004, Kleschevnikov, Belichenko et al. 2012), along with increased GABAergic synaptic density in the inner molecular layer and granular layer of adult Ts65Dn mice (Martinez-Cue, Martinez et al. 2013, Garcia-Cerro, Martinez et al. 2014, Mojabi, Fahimi et al. 2016), while the cerebellar granule cells showed increased excitability and decreased tonic inhibition (Usowicz and Garden 2012, Szemes, Davies et al. 2013). An explanation for these seemingly contrasting results could be the parameters for the analysis, the sex of the animals, the age or the sub-region studied. Of note, the *KCNJ6* gene, encoding for the GIRK channel subunit 2 (GIRK2), is triplicated in Ts65Dn mice leading to mRNA and protein upregulation in hippocampus, cortex and midbrain (Harashima, Jacobowitz et al. 2006).

Studies on DS human brains report reduced brain size and decreased number of neurons due to overall deficits in neurogenesis during development (Contestabile, Fila et al. 2007, Guidi, Bonasoni et al. 2008). Histological studies revealed decreased number of parvalbumin and calbindin positive cells in the frontal and temporal cortex of DS humans (Kobayashi, Emson et al. 1990). Additionally, gene expression analysis of cortical neuronal progenitors obtained from DS humans revealed a decrease in the GABAergic neuron proliferation and changes in the expression of $GABA_A$ receptor subunits (increased GABAAα2 and downregulation of the GABAAα3 and α5) (Bhattacharyya, McMillan et al. 2009). Interestingly, DS people-derived iPSCs show deficits of GABAergic neurons (smaller size, processes and altered migratory pathways) (Huo, Qu et al. 2018), in line with the previous reports (Bhattacharyya, McMillan et al. 2009). Overall, the data from the human studies do not seem to support the data derived from preclinical studies showering increased GABA-mediated transmission due to increased number of GABAergic interneurons (Contestabile, Magara et al. 2017). On the other hand, DS humans and DS animal models show an increased susceptibility to seizures, hyperactivity and anxiety pointing to excess excitation rather than inhibition in DS (Pueschel, Bernier et al. 1991, Stafstrom, Patxot et al. 1991, Escorihuela, Fernandez-Teruel et al. 1995, Reeves, Irving et al. 1995, Goldberg-Stern, Strawsburg et al. 2001, Lott and Dierssen 2010, Westmark, Westmark et al. 2010, Lott 2012, Moss, Richards et al. 2013, Vicari, Pontillo et al. 2013, Deidda, Parrini et al. 2015, Robertson, Hatton et al. 2015, Dekker, Sacco et al. 2018).

Interestingly, DS mice, recapitulate the impairments in cognitive performance characteristic of DS individuals. This has been demonstrated by investigating the molecular substrates underlying learning. For example, altered synaptic plasticity (both LTP and LTD) in hippocampal slices (Siarey, Stoll et al. 1997, Kleschevnikov, Belichenko et al. 2004, Costa and Grybko 2005, Belichenko, Kleschevnikov et al. 2007, Fernandez, Morishita et al. 2007, Kleschevnikov, Belichenko et al. 2012, Belichenko, Kleschevnikov et al. 2015, Deidda, Parrini et al. 2015) was described in DS mice. Given the evidence suggesting a role of altered GABAergic signaling in the cognitive deficits and abnormalities in synaptic plasticity observed in Ts65Dn mice, several studies have evaluated $GABA_A$ receptors as a possible therapeutic target to rescue GABAergic activity and cognitive impairment in DS. A growing body of studies consistently reported that inhibition of $GABA_A$ receptors targeting several subunits rescued LTP and hippocampal-dependent cognitive abilities in Ts65Dn mice (Fernandez, Morishita et al. 2007, Rueda, Florez et al. 2008, Braudeau, Dauphinot et al. 2011, Braudeau, Delatour et al. 2011, Martinez-Cue, Martinez et al. 2013, Potier, Braudeau et al. 2014). Of note, fluoxetine treatment and environmental enrichment, both reported to reduce GABAergic signaling, rescued plasticity and cognitive abilities in Ts65Dn mice (Martinez-Cue, Baamonde et al. 2002, Martinez-Cue, Rueda et al. 2005, Sale, Maya Vetencourt et al. 2007, Maya Vetencourt, Sale et al. 2008, Baroncelli, Sale et al. 2010, Begenisic, Spolidoro et al. 2011, Mendez, Pazienti et al. 2012, Caiati and Cherubini 2013, Begenisic, Baroncelli et al. 2014, Begenisic, Sansevero et al. 2015). The evidence from animal models and human studies support the hypothesis of a causal link between the altered GABAergic transmission, impaired synaptic plasticity and cognitive deficits in DS, highlighting GABAergic signaling as a promising therapeutic target in DS.

Depolarizing $GABA_AR$ signaling in Down syndrome and the role of NKCC1

In 2015, Deidda and colleagues proposed a new link between $GABA_AR$ signaling and cognitive impairments in DS (Deidda, Parrini et al. 2015). Specifically, they found that defective GABAergic activity was mediated by an increased expression of NKCC1 protein in the whole hippocampus (and the CA3-CA1 analyzed separately) and cortex of adult Ts65Dn mice compared to WT animals, without an upregulation of NKCC1 mRNA. This suggested that possibly post-translational modifications underlie NKCC1 protein upregulation. The finding from the animal

model was supported by biochemical analysis of adult human DS hippocampi, revealing an upregulation of NKCC1 also in DS human. Notably, KCC2 protein levels were found unaltered both in Ts65Dn mice and human tissue.

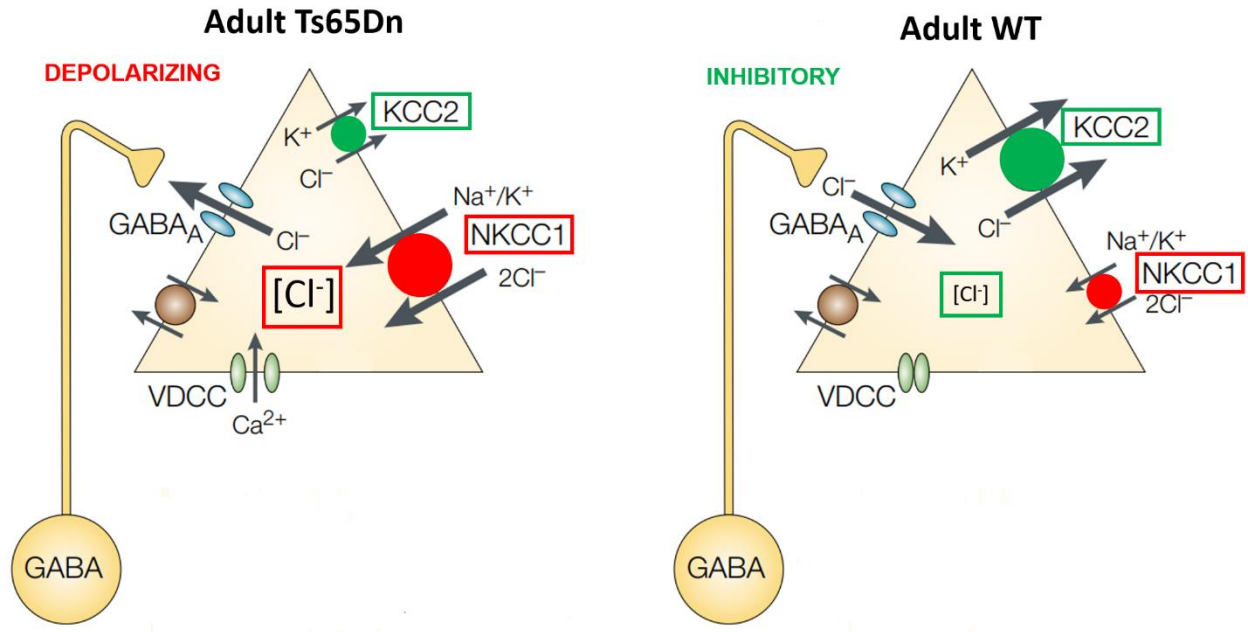


Figure 8. GABA exerts a depolarizing activity in adult Ts65Dn neurons Left, in adult Ts65Dn neurons high NKCC1 expression leads to accumulation of intracellular chloride and upon $GABA_A$ receptor activation depolarization and excitation. Right, in the adult WT neurons the physiological concentration of chloride in the cell determines a hyperpolarization of the membrane upon $GABA_AR$ activation. Adapted from (Ben-Ari 2002).

Given the upregulation of NKCC1 that could lead to changes in the polarity of $GABA_AR$ signaling, the authors investigated GABAergic transmission in adult Ts65Dn mice. In these animals, they found that GABAergic transmission was depolarizing and mostly excitatory rather than hyperpolarizing and inhibitory (Figure 8) (Deidda, Parrini et al. 2015). In particular, they reported the presence of increased GABA-mediated spiking events in the CA1 hippocampal pyramidal neurons in response to exogenous GABA in adult Ts65Dn slices. Accordingly, bath application with bicuculline, an antagonist of $GABA_A$ receptors to block endogenous $GABA_A$ signaling decreased spiking frequency in Ts65Dn neurons.

Deidda and colleagues used the gramicidin-perforated technique for whole-cell recordings, which preserves the endogenous intracellular Cl^- concentration. They found that the reversal potential of GABA-induced currents (ECl^-) of adult Ts65Dn CA1 pyramidal neurons was shifted to more positive values in comparison to the WT neurons (Figure 8). Specifically, Ts65Dn neurons exhibited a more positive ECl^- (-58mV) compared to WT neurons (-66mV) with a resting potential of -62mV. This would predict an outward Cl^- current mediating depolarization in Ts65Dn neuron, and an inward Cl^- current mediating hyperpolarization in WT neurons upon $GABA_AR$ activation. The authors confirmed these results demonstrating increased resting $[Cl^-]i$ in Ts65Dn neurons of the CA1 region by using two-photon imaging with a chloride-sensitive dye. The use of gramicidin perforated patch-clamp was an important step in determining changes in the reversal potential ECl^-. In fact, a study using whole-cell patch-clamp recordings reported no differences in ECl^- in Ts65Dn mice, possibly due to dilution of the intracellular chloride concentration from the pipette solution (Kleschevnikov, Belichenko et al. 2012).

Bumetanide treatment rescues functional and behavioral deficits in Ts65Dn mice

Considering that the increased expression of NKCC1 leads to depolarizing GABAergic transmission and impaired synaptic plasticity in Ts65Dn mice, Deidda and colleagues investigated the pharmacological inhibition of NKCC1 by bumetanide as a potential therapeutic strategy for the cognitive deficits in Ts65Dn animals. Interestingly, bumetanide restored the hyperpolarizing and inhibitory GABAergic signaling in acute hippocampal slices of adult Ts65Dn mice (Deidda, Parrini et al. 2015). Specifically, bath application of bumetanide was able to rescue the altered ECl^- and decreased the GABA-induced spiking events, with no significant phenotype of bumetanide application in WT slices. This suggested that the shift of ECl^- in adult Ts65Dn mice was responsible for the depolarizing $GABA_AR$ activity. Moreover, bumetanide bath application restored the impaired CA1-CA3 LTP in acute brain sliced from adult Ts65Dn mice, with no effect on the LTP found in WT animals (Deidda, Parrini et al. 2015).

Given the evidence in slices the authors proceeded with the *in vivo* evaluation of intraperitoneal bumetanide treatment in WT and Ts65Dn adult animals. All the three bumetanide treatment strategies investigated (acute, sub-chronic-1 week or chronic-4 weeks) rescued hippocampus-

dependent learning and memory deficits in Ts65Dn animals in three independent behavioral tests (object recognition test, object location test and contextual fear conditioning task) all assessing long-term memory in rodents (Figure 9) (Deidda, Parrini et al. 2015).

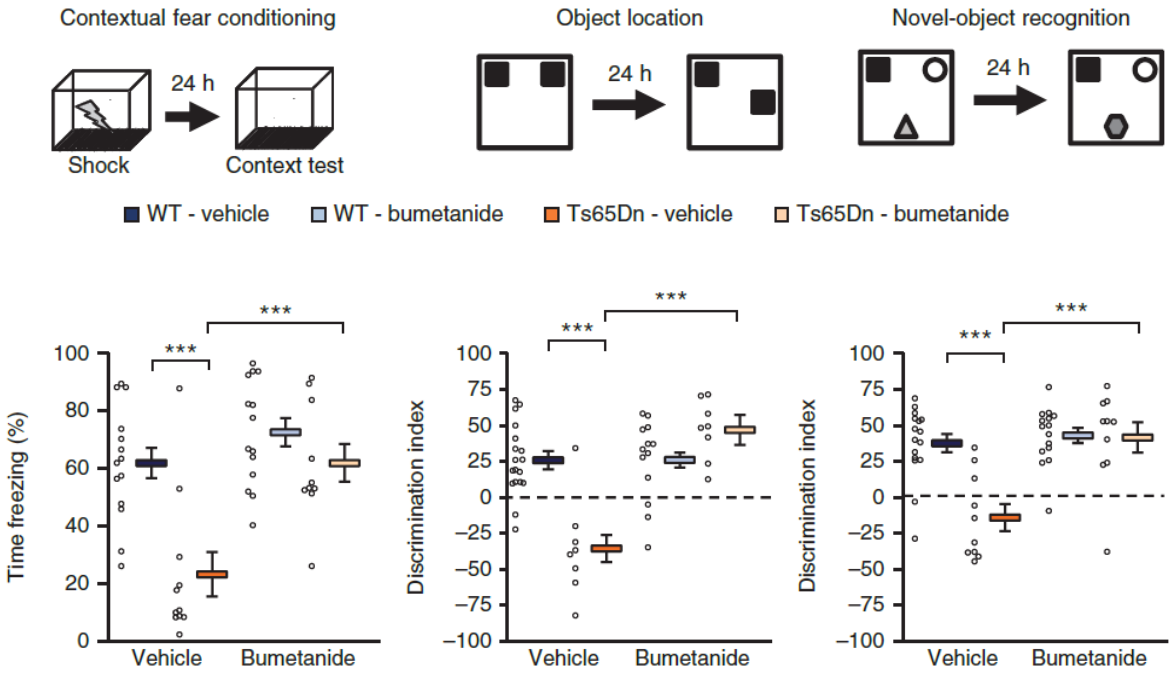


Figure 9. Bumetanide treatment of adult Ts65Dn animals rescues long-term hippocampus-dependent memory deficits. Top, from left to right: Schematic representation of the contractual fear conditioning task, object location task, novel object recognition task. Bottom, from left to right: Quantification of the performance after acute bumetanide treatment in the contractual fear conditioning task, object location task, novel object recognition task. Adapted from (Deidda, Parrini et al. 2015).

Recently, Savardi and colleagues demonstrated that a newly synthetized compound selectively inhibiting NKCC1 over NKCC2 (avoiding in this way the diuretic effect of bumetanide) is able to restore the physiological intracellular Cl^- in Ts65Dn neuronal cultures. Moreover, *in vivo* testing of the NKCC1-selective inhibitor rescued poor short-term and long-term memory in young adult Ts65Dn mice as assessed by T-maze, novel object recognition, object location and contextual fear conditioning task (Savardi, Borgogno et al. 2020). Of note, the new NKCC1-selective inhibitor did not reveal any off-targets in *in vitro* assays and no overt toxic effects were reported in treated animals. These evidence support therapeutic interventions targeting NKCC1 in DS.

Neurodevelopment is dictated by timing and specificity

The pioneering work of Wiesel and Hubel in 1965 (Wiesel and Hubel 1965) opened the way for a new branch of neuroscience investigating the importance of the timing in the development of the brain. The brain is developing in tightly regulated and temporally precise manner. In particular, the assembly of neuronal networks is accomplished by functional feedbacks on time-specific and region-specific developmental processes (Ben-Ari and Spitzer 2010). In the dynamic process of brain development, any time-specific perturbation in the acquisition of the functional neuronal properties that leads to a delay or the failure in the maturation of a given developmental stage can thus leave a pre-symptomatic signature for the development of a neurodevelopmental disorder that might be diagnosed only later in life (Figure 10) (Ben-Ari and Spitzer 2010). Perturbations during brain development can include innate insults (e.g., gene mutations) or environmental insults (e.g. inflammation, early-life-stress, toxins) and might lead to very different functional phenotypic changes, depending on the time or the signaling network that they disrupt (Ben-Ari and Spitzer 2010, Cristino, Williams et al. 2014, Marguet, Le-Schulte et al. 2015, O'Donovan and Owen 2016, Wallace 2016).

Regardless of the underlying cause for the development of an ND, the early onset and the developmental delays in reaching functional milestones are common across syndromic and non-syndromic conditions (Meredith 2015). Emerging data indicate that many neurodevelopmental disorders arise from the alteration of normal developmental trajectories (Marin 2016, Del Pino, Rico et al. 2018, Chorna, Cioni et al. 2020). This is the case also for NDs that are clinically diagnosed when manifest behaviorally years after the first brain alterations, as in the case of schizophrenia (Marin 2016). Interestingly, data deriving from epidemiological, genetic, epigenetic, proteomic and clinical studies suggest the presence of a critical window during brain development for the establishment of either risk or resilience to adult neuropsychiatric disorders (Levitt and Veenstra-VanderWeele 2015). The behavioral manifestation can be further complicated if initial functional changes trigger compensatory mechanisms affecting further developmental processes and structures (Krol and Feng 2018). Future investigations should focus extensively on the brain alterations induced by innate or environmental insults that occur during the dynamic brain development linking the gap between time/brain region and ND symptomatology. The very existence of these critical windows of brain susceptibility might be a

good starting point for the understanding of the overlapping clinical features present in many neurodevelopmental disorders (Chisholm, Lin et al. 2015, Ghiani and Faundez 2017). For example, mild to severe intellectual disability, language and social communication deficits, repetitive behavior, epilepsy and hyperactivity are commonly present in many NDs (Owen 2012, Harris 2014).

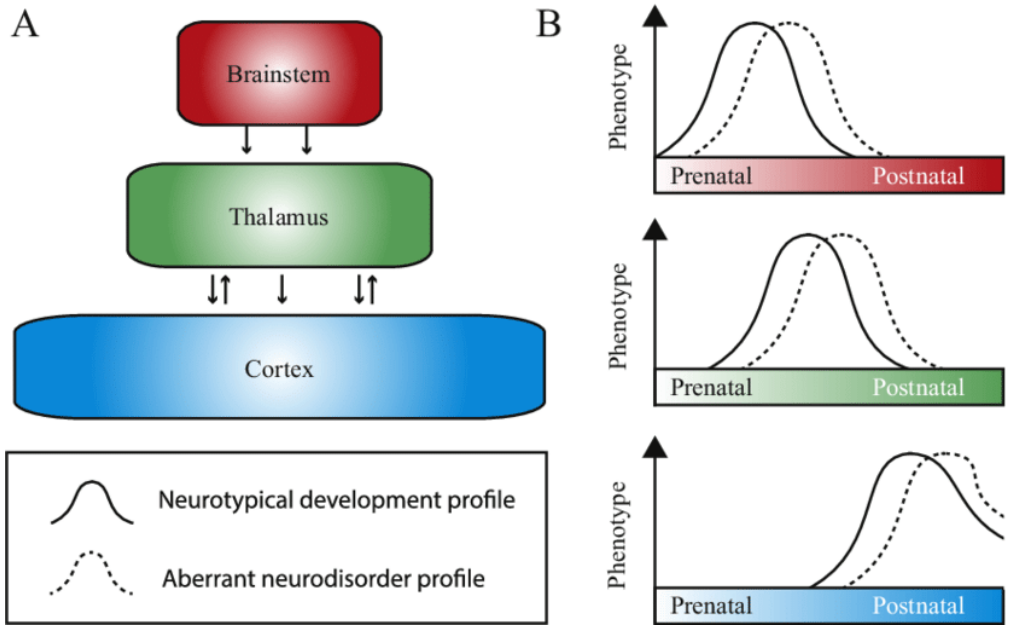


Figure 10. Critical periods across brain regions and the sequential disruption in NDs. (A) Representation of the evolutionary caudal to rostral maturation for each brain region (brainstem, thalamus, cortex), where functional monosynaptic connections form from one region to the next one. **(B)** The full line represents the neurotypical opening and closure for the critical period of plasticity for each region. The dashed line represents aberrant development potentially due to a delay. Phenotype refers to any kind of structural and functional changes that differ between neurotypical and aberrant profiles due to timing. Adapted from (Meredith 2015).

Timing is an important parameter for the effectiveness of therapeutic interventions in NDs

Pathological development is a dynamic process demanding personalized interventions with implementation onset as early as possible (Lazaratou, Economou et al. 2017). It is increasingly evident that the greater level of brain plasticity during critical periods provides an opportunity for

better and long-lasting response to therapeutic interventions with early onset of rehabilitation (Johnston 2009).

The concept of early behavioral interventions in pathological development has been present also in traditional psychiatry. However, genetic and pharmacological therapeutic interventions in NDs have been investigated to a great extent only in the very last years. Studies in animal models of NDs confirm the hypothesis that altered cellular phenotypes exist long before clinical behavioral deficits are manifested (Krol and Feng 2018). Accordingly, early interventions in these animal models have often been more effective in ameliorating the burden of behavioral deficits than later interventions, when the critical periods of brain plasticity are closed. (Krol and Feng 2018).

Given the important role that GABAergic signaling has in brain development, it is not a surprise that many of the studies discussed above on NDs report alterations and investigate therapeutic interventions targeting the GABAergic system. In particular, in 2015, Marguet and colleagues linked the alteration observed in cortical and hippocampal network activity in the neonatal Kv7-deficient mouse model of genetic epilepsy to structural and behavioral impairments that they found in the adult life (Marguet, Le-Schulte et al. 2015). They reported that pharmacological intervention with bumetanide during the first two weeks of life, a period of aberrant neuronal activity, could prevent the development of pathological network activity, along with structural and epileptic phenotype in the adulthood. Interestingly, the authors did not report any adverse effect of early bumetanide treatment, and no significant phenotype of bumetanide in control mice. The same group previously (Peters, Hu et al. 2005) reported that restoration of Kv7 channel function in young adults did not rescue the adult phenotype in the animals. The restricted temporal window of effective intervention demonstrates that identifying a target period is an essential component of treatment (Krol and Feng 2018).

Recent studies have also highlighted that early therapeutic interventions can have a long-lasting structural and functional impact on the brain of Fragile X mice. In particular, Fragile X mice (*Fmr1* KO mice) was shown to have high expression of NKCC1 protein (but not mRNA) in the cortex at P10 leading to a delay of the GABA polarity switch from depolarizing to hyperpolarizing. This contributes, at least to some extent, to the pathological synaptic phenotype in the *Fmr1* KO cortex (He, Nomura et al. 2014). Interestingly, bumetanide treatment during early postnatal life (P0-P10) rescued LTP in the somatosensory cortex and restored whisker evoked-response in the adult *Fmr1*

KO mice. That was followed by a proteome remodeling in the barrel cortex of treated animals. The most notable findings of this study were restoration of MeCP2 and GAP43 expression in bumetanide-treated *Fmr1* KO mice compared to controls *Fmr1* KO, and increase in the levels of parvalbumin (PV) and TrkB after bumetanide treatment (He, Arroyo et al. 2019). In line with these data, Dansie et al. showed that oral minocycline treatment (from birth until weaning) reduced locomotor activity and partially rescued audiogenic seizures in young *Fmr1* KO mice. The long-term rescue was possible only when treatment was given during development and not in the adult life, when constant treatment was needed for the rescue of the phenotype (Dansie, Phommahaxay et al. 2013). In addition, a single induction of epileptic seizure at P10 can lead to long-term behavioral defects in the adult *Fmr1* KO mice, again stressing the importance of time-specific perturbations in brain development (Hodges, Reynolds et al. 2019). Moreover, Tyzio and colleagues found decreased level of KCC2 in the hippocampi of autistic mice at P15 and P30 causing alteration of GABAergic transmission. That was corrected -along with behavioral autism-like features later in life- by maternal treatment with bumetanide right before birth (Tyzio, Nardou et al. 2014). The beneficial effects of early intervention has been also demonstrated in 2 young children with Fragile X syndrome. Psychopharmacological treatments with (memantine, sertraline, minocycline as added in this order), when combined with intensive educational training, improved their cognitive and behavioral performance (Winarni, Schneider et al. 2012).

Early alterations have been reported also in Rett syndrome. A recent study on Mecp2 mutant mice showed that the developmental GABAergic shift is abolished at birth in CA3 pyramidal neurons of Mecp2 mutant mice, and that, two weeks later, GABA exerts strong excitatory activity (Lozovaya, Nardou et al. 2019). Maternal bumetanide treatment one day before delivery rescued GABAergic activity (inhibitory GABA, spontaneous glutamatergic and GABAergic activity, and metabotropic LTD) two weeks after, but it did not rescue respiratory or weight deficits (Lozovaya, Nardou et al. 2019).

In Smith–Magenis syndrome, a neurodevelopmental disorder that is caused by loss of one copy of the *RAI1* gene, conditional reactivation of *RAI1* only in young animals (3-4 weeks old), but not later (7-8 weeks) after birth rescued social deficits. Of note, in the *Scn1a*$^{+/-}$ mouse model of Dravet syndrome aberrant parvalbumin-interneuron activity during the first weeks of life contributes to

chronic epilepsy onset in the adulthood even if aberrant interneuron activity is normalized by P35 (Favero, Sotuyo et al. 2018).

Of note, exposure to cannabinoids during lactation delays the upregulation of KCC2 and thus the switch of GABA polarity in the mPFC, along with alterations in ultrasonic vocalizations in rats. Notably, bumetanide treatment corrected the delayed GABA switch. By co-administering a CB1R antagonist the authors confirmed that the effects of early cannabinoid exposure are CB1R-mediation (Scheyer, Borsoi et al. 2020).

In humans, the first-line treatment of choice for attention-deficit/hyperactivity disorder (ADHD) is methylphenidate, a norepinephrine–dopamine reuptake inhibitor (NDRI). Interestingly, imaging data show that the effectiveness of the treatment in enhancing GABA basal levels (reduced in the prefrontal brain regions of ADHD people) is age-dependent (Solleveld, Schrantee et al. 2017). In particular, when the treatment is given at a young age (before 16 years old) the changes in the GABAergic system are lasting in adult life, while first treatment response in adulthood (> 23 years) exerts no major effects demonstrating that treatments with early onset have better outcomes that treatments administered later in life (Solleveld, Schrantee et al. 2017). In humans, the concept of very early interventions is the focus of recent clinical trials focusing on deficit-targeted interventions within the first year of life for preterm babies (Kooiker, van der Linden et al. 2020). The trials integrate a collaborative approach between experts and families along with recent technological advances that enabled the use of online tools for tele-monitored home intervention in infancy, with positive outcomes in motor and visual development of preterm infants later in life (Rollins, Campbell et al. 2016, Sgandurra, Bartalena et al. 2016, Coufal and Woods 2018, Chung, Donelan et al. 2021). Other early interventions in preterm babies are being carried out also using ergo therapy and physiotherapy interventions with positive acute outcomes (Kepenek-Varol, Tanriverdi et al. 2019), but further studies need to evaluate the long-term effects of early physiotherapy approaches to preterm babies.

Finally, it is important to be noted that in adult life genetic and pharmacological manipulations in animal models of neurodevelopmental disorders can still rescue several alterations that arose during pathological development at molecular, network activity and behavioral level (Ehninger, Li et al. 2008, Castren, Elgersma et al. 2012). However, many interventions require chronic administration of the therapeutic intervention for the rescue of the deficits (Dansie, Phommahaxay

et al. 2013, Deidda, Parrini et al. 2015, Pinto, Morelli et al. 2020). Moreover, many deficits cannot be rescued by therapeutic interventions in the adult life, indicating that earlier intervention are needed at the time the deficits are encoded in the circuits (Clement, Aceti et al. 2012, Deidda, Parrini et al. 2015, Mei, Monteiro et al. 2016, Solleveld, Schrantee et al. 2017, Krol and Feng 2018). Indeed, the windows of enhanced plasticity during brain development offer opportunity windows for the rescue of pathological developmental trajectories, conferring long-lasting effects with target and time specific interventions (Johnston 2009, Lazaratou, Economou et al. 2017, Krol and Feng 2018).

Timed therapeutic interventions in Down syndrome

In the case of Down syndrome, as in other NDs, many (but not all) brain alterations arise during the development. Interestingly, recent MRI volumetric analyses of Down syndrome fetuses and neonates reveal alterations from typically developed euploid infants (Patkee, Baburamani et al. 2020). The first alterations are described during the second trimester of pregnancy in the cerebellum and during the third trimester for the cortex. A similar study with a larger cohort described similar alterations with decreased growth trajectories of the cortical plate, the subcortical parenchyma, and the cerebellum in DS fetuses (Tarui, Im et al. 2020). These alterations in the size of diverse brain regions are hypothesized to be the structural substrates for later cognitive functional impairments (Rathbone, Counsell et al. 2011, Chorna, Cioni et al. 2020, Patkee, Baburamani et al. 2020).

In line with early brain deficits in DS, some studies demonstrated that early interventions are promising also in DS. Interestingly, early environmental enrichment in Ts65Dn pups during development led to increased maternal care and rescue of synaptic plasticity and cognitive performance in the adulthood (Begenisic, Sansevero et al. 2015). Notably, environmental enrichment in adult Ts65Dn mice was sufficient to promote learning, memory and visual acuity rescue (Martinez-Cue, Rueda et al. 2005, Begenisic, Spolidoro et al. 2011), although the effect was different between sexes in the different tasks performed (Martinez-Cue, Baamonde et al. 2002).

Additionally, a number of studies have highlighted the importance of early (embryonic, perinatal and postnatal) pharmacological interventions for the rescue of structural and functional deficits seen in Ts65Dn mice. In particular, prenatal treatment (between E8-12) with the neuroprotective peptides (NAP+SAL, active fragments of ADNP and ADNF) rescued the motor and sensory milestones reached within 2 postnatal weeks in Ts65Dn mice (Toso, Cameroni et al. 2008), and also prevented learning deficits in adult life (Incerti, Horowitz et al. 2012). Moreover, a single injection of SAG1.1, a synthetic activator of Sonic hedgehog pathway at P0 rescued structural and cognitive deficits in the adult Ts65Dn mice (Roper, Baxter et al. 2006, Das, Park et al. 2013). Notably, maternal choline supplementation (from embryonic life until P21) improved spatial memory performance and neurogenesis in the offspring in the adult life of Ts65Dn mice (Moon, Chen et al. 2010, Velazquez, Ash et al. 2013, Ash, Velazquez et al. 2014, Kelley, Powers et al. 2014, Alldred, Chao et al. 2019, Kelley, Ginsberg et al. 2019). Additionally, embryonic to early postnatal treatment rescued long-term memory and synaptic deficits by increasing plasticity markers (BDNF and phosphorylated CREB) both in three and seven month old Ts65Dn mice (Kazim, Blanchard et al. 2017). Furthermore, a number of studies have investigated interventions targeting the serotonergic system to rescue impaired neurogenesis and dendritic development in trisomic animals. Prenatal and neonatal fluoxetine treatment (a selective serotonin reuptake inhibitor) in Ts65Dn mice during development (P3 to P15) restored hippocampal neurogenesis and rescued memory performance, suggesting long-term effects of early treatment one or three months after treatment cessation (Bianchi, Ciani et al. 2010, Guidi, Stagni et al. 2013, Guidi, Stagni et al. 2014, Stagni, Giacomini et al. 2015). Interestingly, fluoxetine treatment to adult animals does not improve spatial learning and memory impairments and has adverse effects (seizures and mortality) in Ts65Dn mice (Heinen, Hettich et al. 2012). Of note, a study stressed the prophylactic role of the ciliary neurotrophic factor (CNTF) in brain development in Ts65Dn mice.

The studies above clearly demonstrate that early pharmacological or environmental interventions in DS mice during a specific temporal windows of the developing brain can have a positive, long-lasting effects on brain structural and behavioral deficits in adulthood. The magnitude and striking persistence of the effects of neonatal and prenatal interventions emphasizes the importance of early treatment in DS (Stagni, Giacomini et al. 2015). Considering the important role of GABAergic activity in the developing brain the hypothesis of altered GABAergic signaling as causative of the aberrant neural circuit formation in the developing trisomic brain should be investigated

extensively. Interestingly, fluoxetine and environmental enrichment has been both demonstrated to reduce GABAergic signaling (Martinez-Cue, Baamonde et al. 2002, Sale, Maya Vetencourt et al. 2007, Maya Vetencourt, Sale et al. 2008, Baroncelli, Sale et al. 2010, Begenisic, Spolidoro et al. 2011, Begenisic, Sansevero et al. 2015, Baroncelli, Cenni et al. 2017). Thus, the brain structural and behavioral deficits reported rescued in the studies above (Stagni, Giacomini et al. 2015) could be at least partly due to modulation of GABAergic activity. Notably, a first evidence for alterations in the switch of GABA polarity during development was demonstrated recently in Ts65Dn mice (Lysenko, Kim et al. 2018). Here, in primary hippocampal cultures and acute slices from Ts65Dn animals authors demonstrated that GABA polarity switch is delayed in Ts65Dn hippocampus and hypothesize that this prolongation in GABA activity maturation can contribute, to some extent, to altered neuronal circuits in DS (Lysenko, Kim et al. 2018).

RATIONAL OF THE STUDY

Deidda and colleagues reported that in adult Ts65Dn mice the $GABA_AR$ signaling is depolarizing and excitatory due to upregulation of NKCC1 (Deidda, Parrini et al. 2015). Intraperitoneal bumetanide treatment (or NKCC1 inhibition by a selective inhibitor; Savardi, Borgogno et al. 2020) rescued hippocampus-dependent learning and memory deficits in adult Ts65Dn animals in three independent behavioral tests assessing long-term memory in rodents (Deidda, Parrini et al. 2015). However, the effect of bumetanide was not long-lasting, as the positive effects of bumetanide were lost after one week of drug withdrawal, as drug withdrawal experiments revealed (Deidda, Parrini et al. 2015). Moreover, bumetanide treatment in adult Ts65Dn animals did not rescue hyperactivity phenotype and susceptibility to epileptic seizures (Figure 11). Altogether, these results, suggest that bumetanide treatment in adulthood did not induce neuronal rewiring to cause the rescue of cognitive function in adult Ts65Dn mice. Moreover, hyperactivity and increased susceptibility to seizures might in fact depend on mechanisms other than depolarizing GABA signaling or they directly depend on miswiring during development. Here, I will study whether alterations of the transporters (NKCC1 and KCC2) determining GABAergic activity are present since early in development in trisomic animals. I will investigate any long-term effects of early-in-life genetic and pharmacological interventions targeting NKKC1 to rescue deficits in the adult life in Ts65Dn mice using a neuron-specific AAV9-mediated NKCC1 knockdown and bumetanide treatment during the first 2 weeks of development, respectively. This project aims at studying the effectiveness and safety of time-specific interventions for DS. Targeted and timed interventions possibly impacting on the trajectories of the developing brain might offer a promising alternative for long-lasting positive outcomes avoiding the adverse effects of chronic adult treatment.

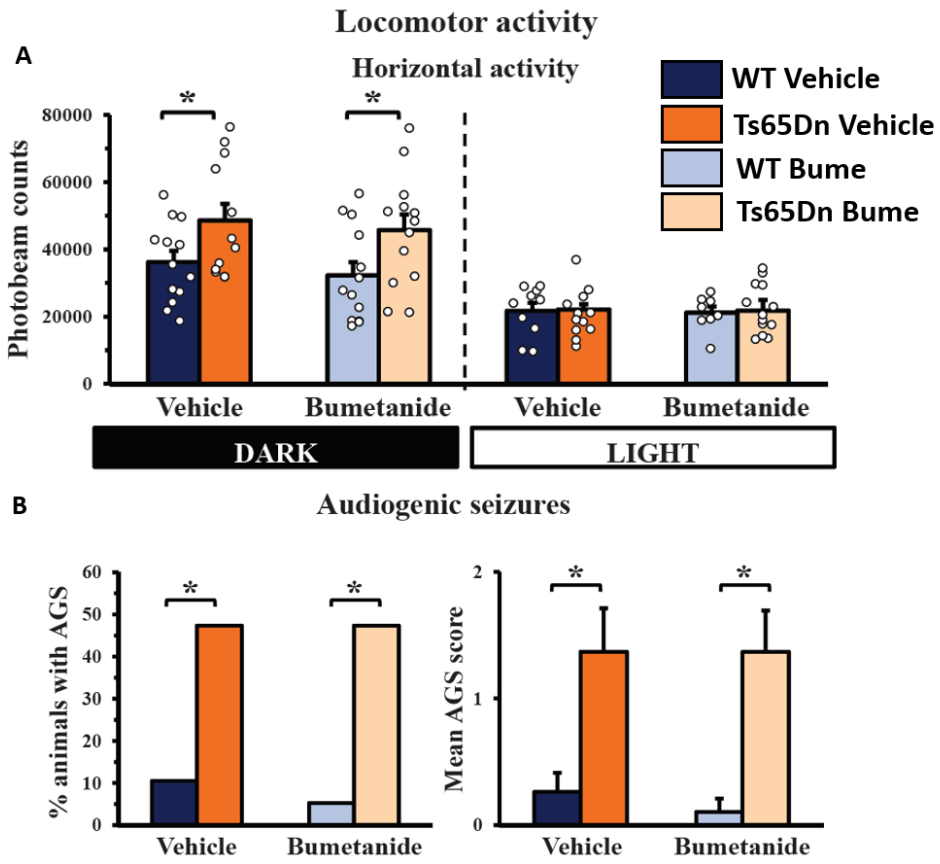


Figure 11. Bumetanide treatment in adult Ts65Dn mice do not rescue hyperactivity and susceptibility to seizure phenotypes. (A) Locomotor activity during dark phase (left) and light phase (right) over the course of 24 hours (divided 12h of light and 12 of dark) in WT and Ts65Dn mice treated with vehicle and bumetanide. **(B)** Susceptibility to seizures as assessed by the audiogenic seizures test in animals treated with vehicle and bumetanide. Left, percentage of animals that exhibited AGS, right, mean AGS severity score. Adapted from (Deidda, Parrini et al. 2015).

Results

1. Altered protein expression of Cl^- transporters in early postnatal life of Ts65Dn mice

To address the developmental profile of NKCC1 and KCC2 of WT and Ts65Dn animals, we dissected their brains and collected the hippocampal areas at different time points spanning from postnatal day 2 (P2) to adulthood. We performed western blot analysis with specific NKCC1 and KCC2 antibodies.

We found that both NKCC1 and KCC2 levels increased with increasing developmental ages, with KCC2 upregulation being many-fold higher than the increase of NKCC1 expression (see scale on y-axis in Figure 13 A,B) in both WT and Ts65Dn animals. Quantification of protein expression in <P60 (adult) hippocampi in WT and Ts65Dn hippocampi (Figure 14).

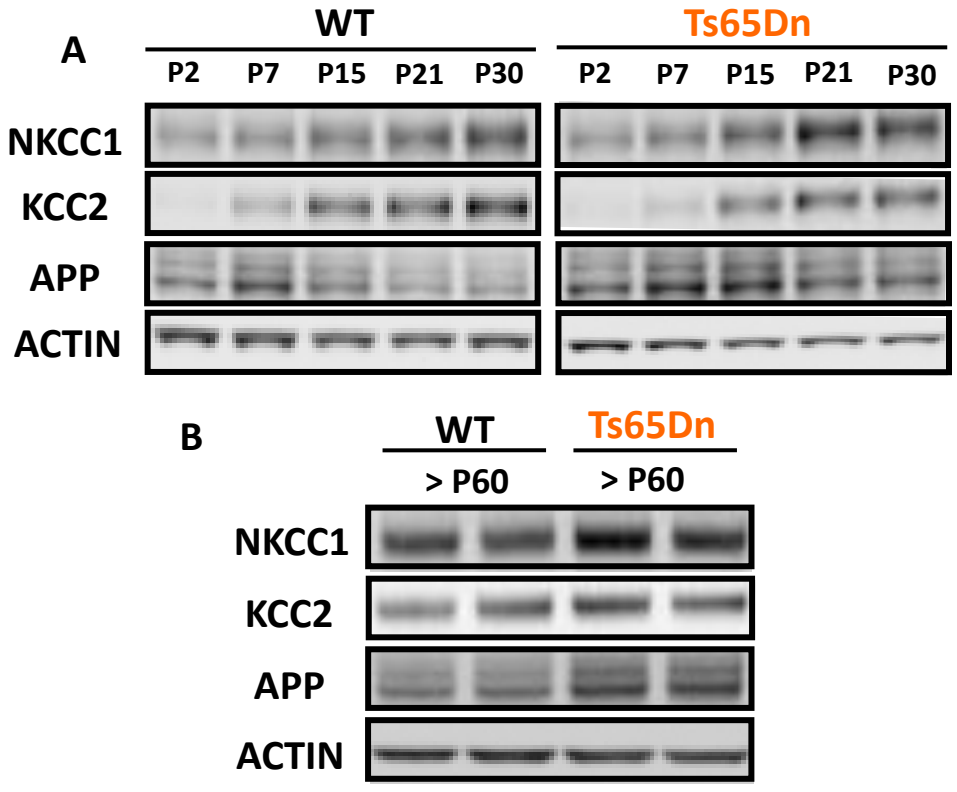


Figure 12. Equal amounts of protein homogenate from hippocampus were loaded onto each lane and probed with antibodies against NKCC1, KCC2, APP and ACTIN. **(A)** Mouse hippocampal samples from different developmental time points. **(B)** Hippocampal samples from adult WT and Ts65Dn mice loaded in equal protein amounts. Actin was used as a housekeeping gene. APP was used as a positive control for the genotype, as APP gene is a triplicated gene in Ts65Dn mice.

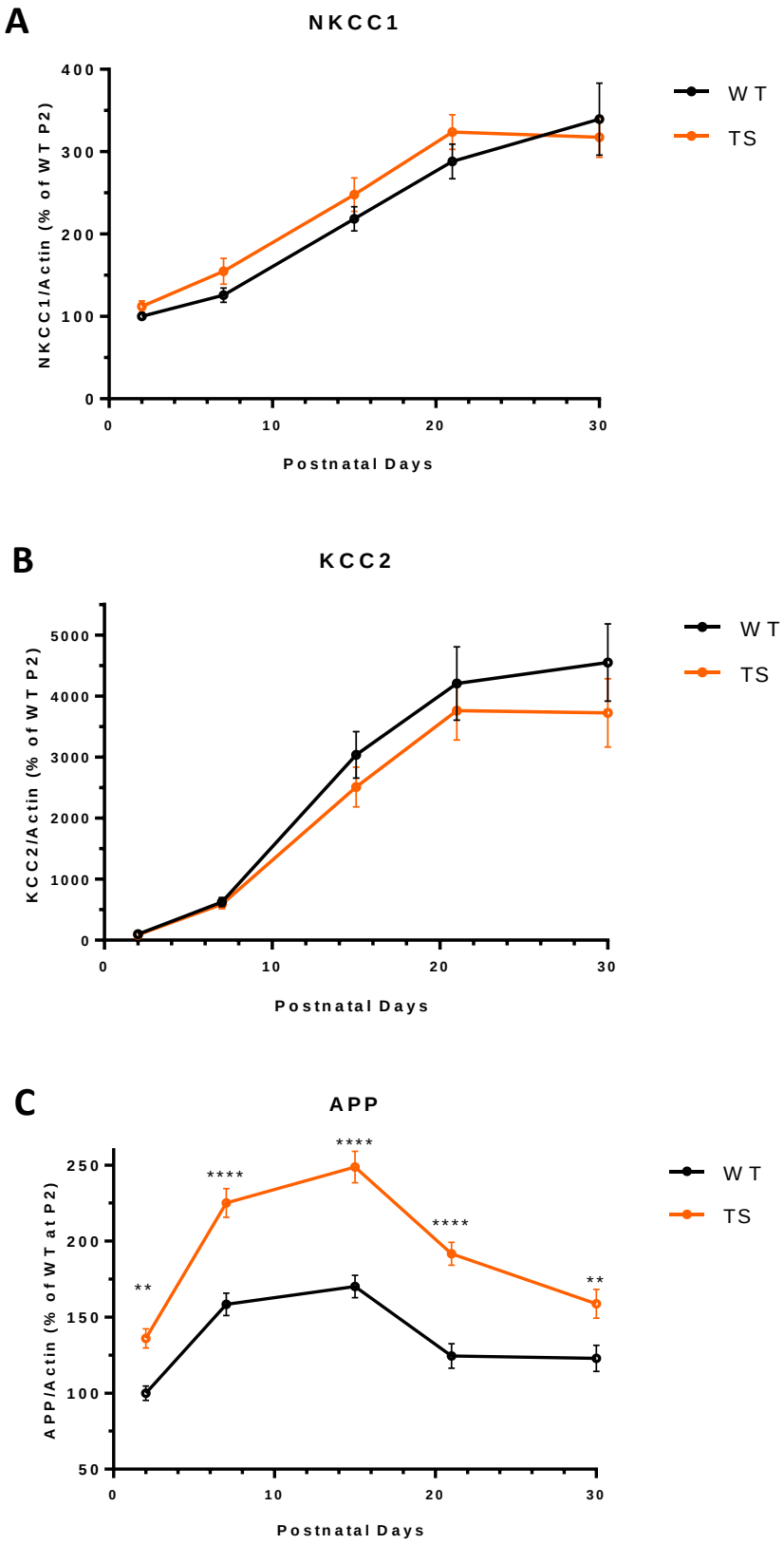

A
NKCC1
NKCC1/Actin (% of WT P2)
400
300
200
100
0
0
10
20
30
Postnatal Days
WT
TS
B
KCC2
KCC2/Actin (% of WT P2)
5000
4000
3000
2000
1000
0
0
10
20
30
Postnatal Days
WT
TS
C
APP
APP/Actin (% of WT at P2)
250
200
150
100
50
0
10
20
30
Postnatal Days
**
****
****
****
**
WT
TS

Figure 13. Quantification of NKCC1 **(A)**, KCC2 **(B)** and APP **(C)** protein expression in the hippocampi of WT and trisomic mice. The results were normalized to the levels of the average of WT brains at P2 (postnatal day 2). **(A)** Circles represent the average protein expression for each time-point ± SEM, (two-way ANOVA, $F_{Interaction}$ (4.152)= 1.004, p = 0.4071, Holm–Sidak post hoc test). **(B)** Circles represent the average protein expression for each time-point ± SEM, (two-way ANOVA, $F_{Interaction}$ (4.152)= 0.7208, p = 0.5790, Holm–Sidak post hoc test). **(C)** Circles represent the average protein expression for each time-point ± SEM, (two-way ANOVA, $F_{Interaction}$ (4.104)= 6.443, p = 0.0001, Holm–Sidak post hoc test, ** p < 0.01, **** p < 0.0001 two-way).

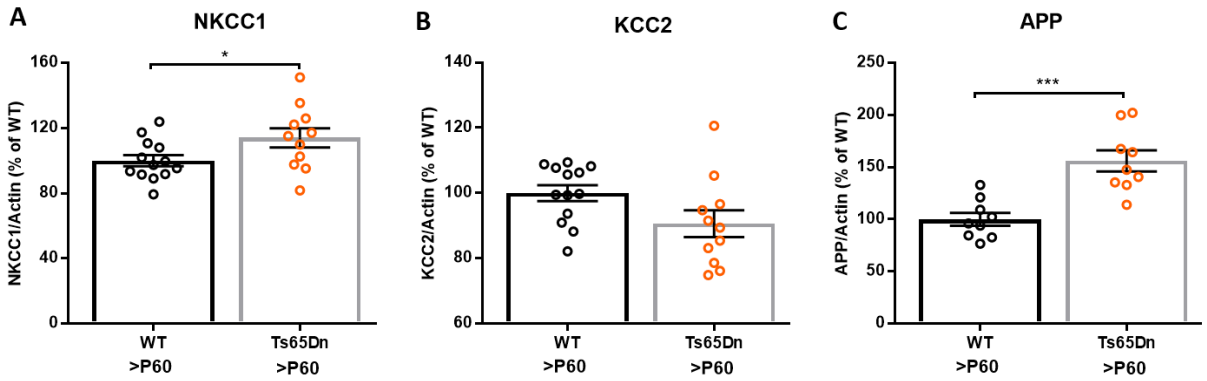


Figure 14. Quantification of NKCC1 **(A)**, KCC2 **(B)** and APP **(C)** protein expression in the hippocampi of >P60 WT and trisomic mice. The results were normalized to the levels of the average of WT. Bars represent the average expression levels ± SEM, and circles represent data points for each animal. Unpaired two-tailed Student's t test (*p < 0.05, ***p<0.001).

In particular, when normalized to the WT levels for each time point studied, Ts65Dn levels for NKCC1 were significantly higher at P7, P21 and >P60 (Figure 15A). Moreover KCC2 levels in Ts65Dn mice where significantly lower at P2, P15 and at P30 (Figure 15B). The results indicate an altered cotransporter expression in trisomic hippocampi since early in postnatal development. For all analysis, we ran a parallel quantification of the APP protein levels, which used as a positive control due to its triplication (and consequent upregulation) in Down syndrome (Figure 15C).

Notably, when we calculated the NKCC1 /KCC2 ratio and normalized it to WT levels, we found that trisomic mice showed significantly high NKCC1 to KCC2 ratio at all developmental points analyzed and in adulthood (Figure 16).

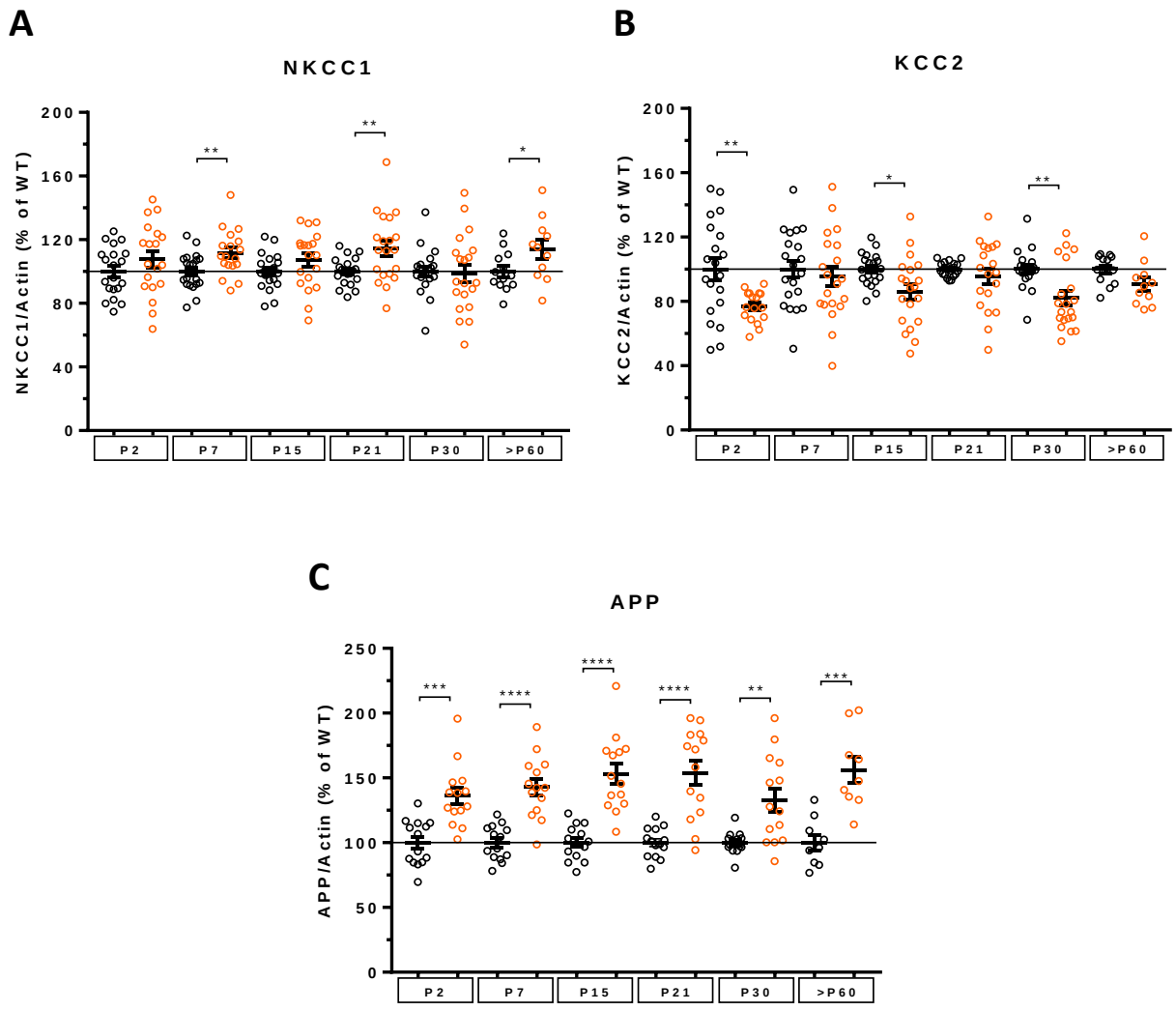


Figure 15. Quantification of Western blots for NKCC1 **(A)**, KCC2 **(B)** and APP **(C)** protein expression in the hippocampi of WT and Ts65Dn mice normalized on Actin. Each time point is normalized in each WT P (postnatal day). Lines report the average protein expression of all analyzed animals (± SEM) and circles represent data points for each animal. Unpaired two-tailed Student's t test (*p < 0.05, **p < 0.01, ***p<0.001) was performed for each time point individually.

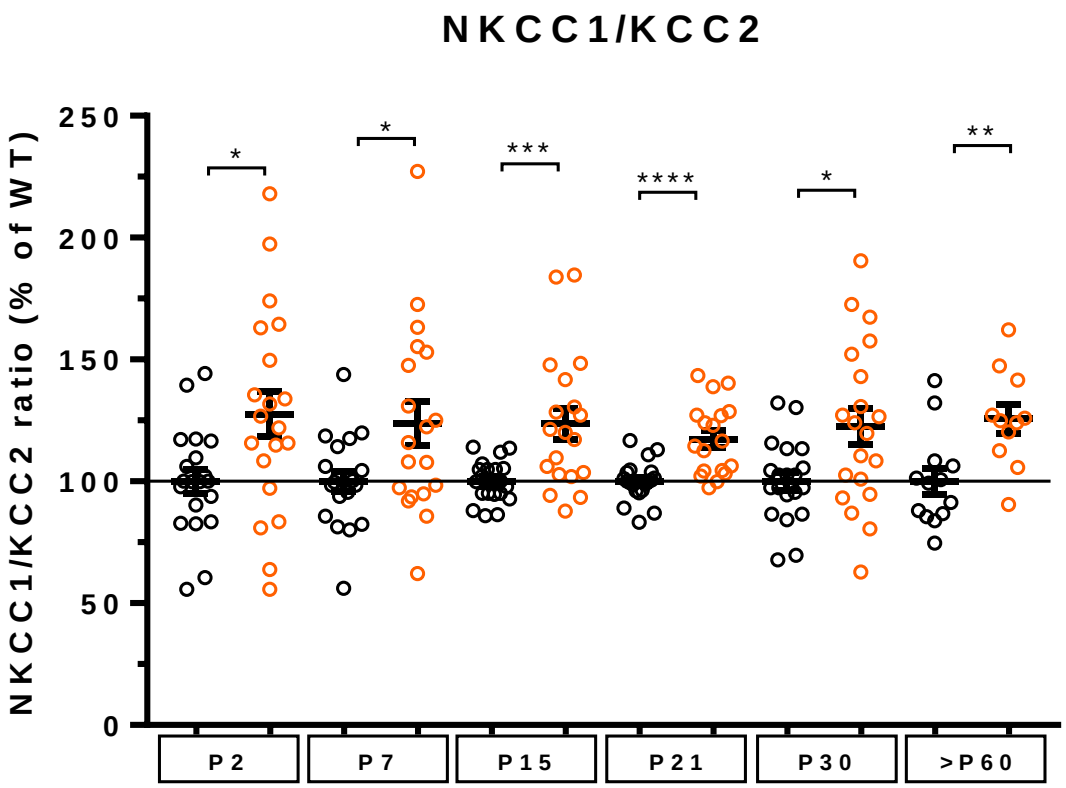


Figure 16. Quantification of the ratio of NKCC1/KCC2 protein expression in the hippocampi of WT and Ts65Dn mice in Western blots experiments. Ts65Dn protein expression level for each time point is normalized to the expression levels of the average of WT samples at the same time point. Lines report the average protein expression of all analyzed animals (± SEM) and circles represent data points for each animal. Unpaired two-tailed Student's t test (*p < 0.05;**p<0.01;*** p<0.001) was performed for each time point individually.

2. *In vitro* validation of viral NKCC1 RNA interference

The early-in-life brain alterations characteristic of the brain of fetuses with Down syndrome are hypothesized to be the structural and molecular substrates for later neurocognitive impairments characteristic of the syndrome (Patkee, Baburamani et al. 2020). Following this rationale and supported by our results showing altered NKCC1/KCC2 ratio present since the first postnatal days of life in trisomic mice, we aimed to test the hypothesis that interfering with the NKCC1 levels early in brain development we could rescue the pathological developmental trajectories possible substrate of behavioral deficits in DS.

To this aim, we employed a tool that was created by the Cancedda lab for RNA interference and consequent reduction of NKCC1protein levels by the use of artificial microRNAs (amiRs) (Figure 17). In particular, amiRs are constructed by replacing the 21-22 nucleotide antisense targeting sequence (the so-called guide strand) of a naturally-occurring primary-microRNA (pri-miRNA) with an antisense sequence against hNKCCl. This RNA-interference tool, achieves neuron-specific expression of the amiR against NKCCl by using a human Synapsin promoter to drive the transgene expression. This invention has been submitted for patent application under the publication number WO/2018/189225. Notably, as commonly used for research purposes in recombinant AAV vectors (rAAV), the two viral open reading frames (ORF) *rep* and *cap*, naturally being flanked between ITR they are inserted as helper genes *in trans* to package the transgene inside the capsid and the gene of interest is inserted in their place (Kwon and Schaffer 2008). In this way the ability of recombinant AAV to integrate into its preferred site of genomic integration termed as AAVS1 is abolished (Deyle and Russell 2009). The recombinant AAV serotype 9 we used for our study is expressed throughout life episomally without integrating in the host genome (Penaud-Budloo, Le Guiner et al. 2008).

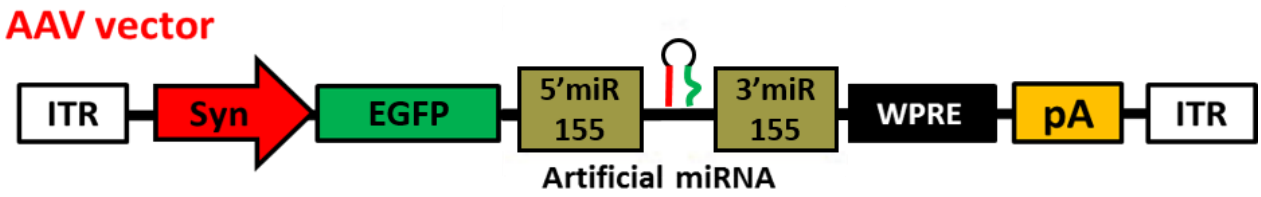


Figure 17. Schematic representation of the AAV9 construct bearing the artificial miRNA (amiR) against NKCC1. AAV9 vector is under the human Synapsin promoter (syn) for selective expression of the construct in neurons. EGFP is a reporter gene to monitor transduction of the virus followed by the artificial miRNA against NKCC1. WPRE (woodchuck hepatitis post-transcriptional regulatory element) is for enhancement of the stability of the viral transcripts. Polyadenylated 3′-end signal (pA) is for transcription termination. ITR (inverted terminal repeat) flanked transgene expression cassette to enhance gene expression. The red and green in the sequence loop indicates the 5′-flanking and 3′-flanking regions, respectively.

First, we tested the AAV9 vector in primary neuronal cultures. The amiR against NKCC1 significantly downregulated NKCC1 protein levels (Figure 18B) compared to the control vector. Moreover, the vector was expressed only in neurons and not in astrocytes, in agreement with its neuron-specific synapsin promoter (Figure 18A).

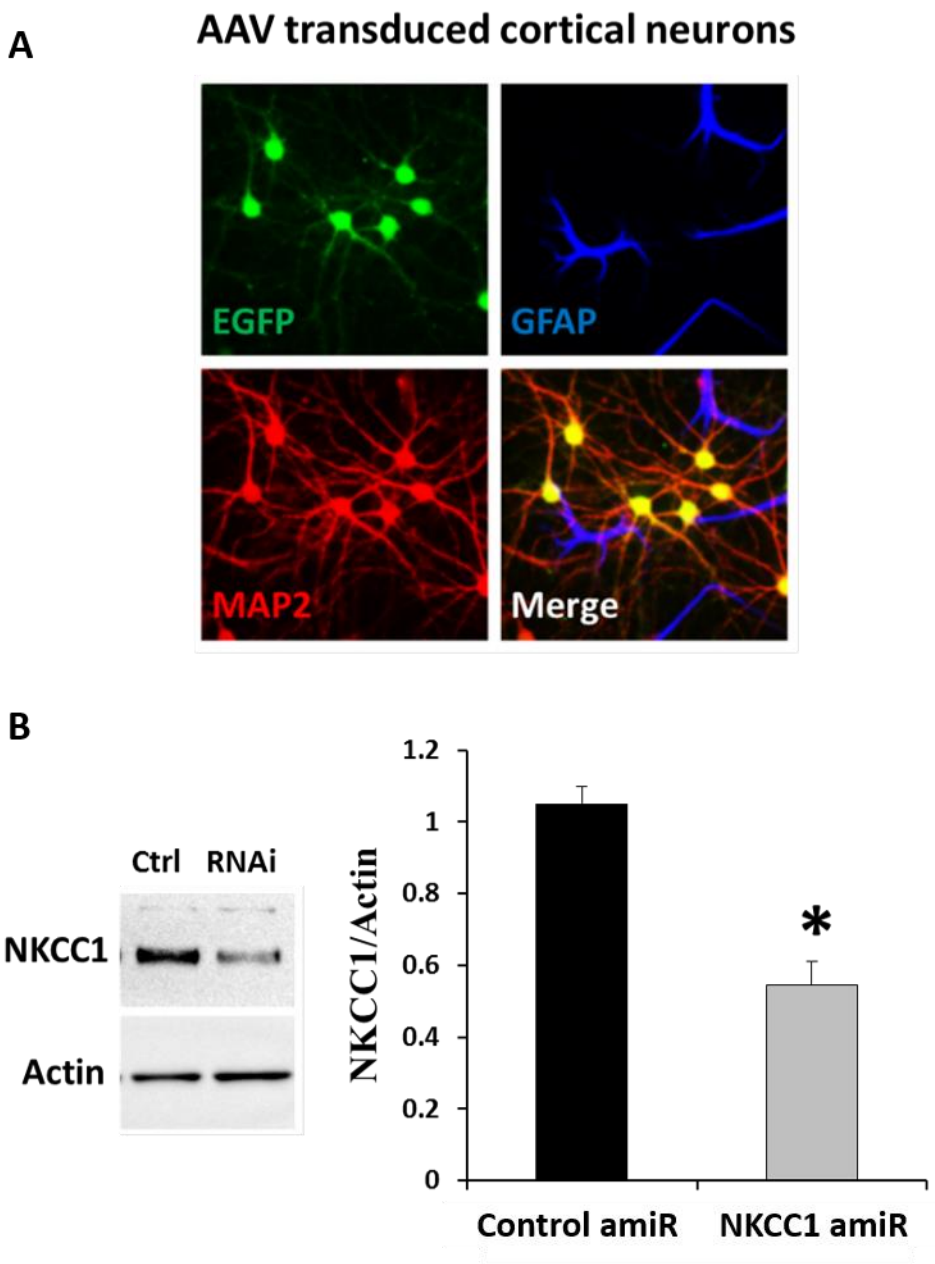


Figure 18. *In vitro* validation of AAV9 viral construct for RNAi interference against NKCC1 under the Synapsin promoter in primary cortical neuronal cultures. **(A)** Primary cortical cultures at DIV 7 transduced with the AAV9 construct expressing GFP. GFP shows co-localization with the specific neuronal MAP2 and does not co-localize with the astrocytic marker GFAP. **(B)** Western blot analysis from cortical cultures transduced with the AAV construct with artificial miRNA (amiR) targeting NKCC1. Unpaired two-tailed Student's t test (*$p < 0.05$).

3. GFP expression mediated by early-in-life AAV9 infection is present in large transduction brain-areas in adulthood

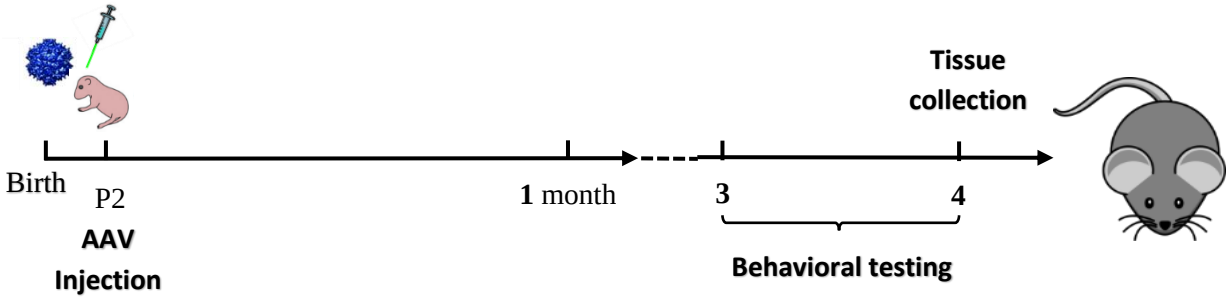
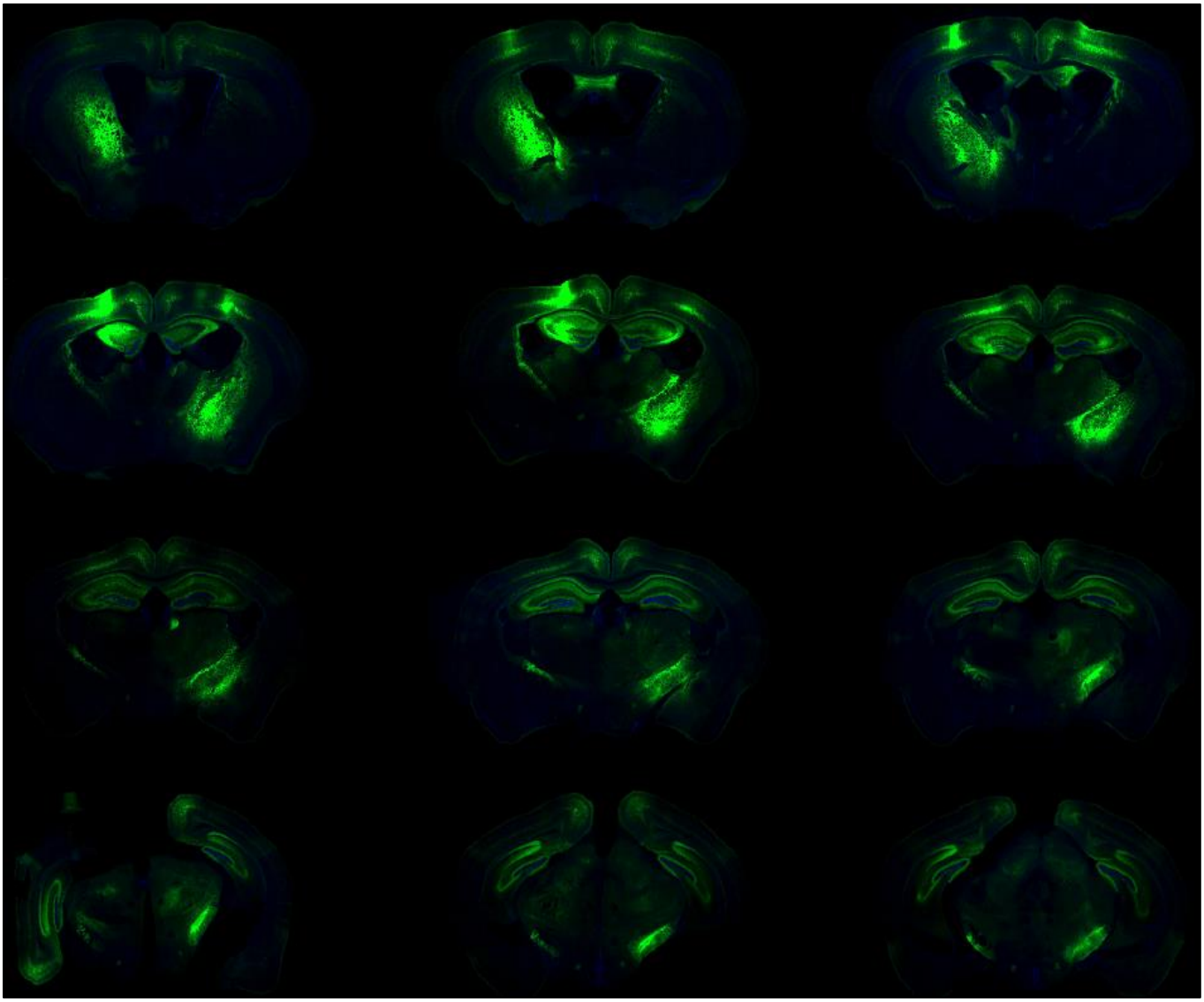


Figure 19. Schematic representation of the experimental protocol for the *in vivo* experiments. Bilateral intraventricular injection with the AAV9 construct was performed at postnatal day 2 in WT and Ts65Dn pups. Behavioral testing was performed between P90-120 (see Figures 9-14) and tissues collected at the end of the experiment.



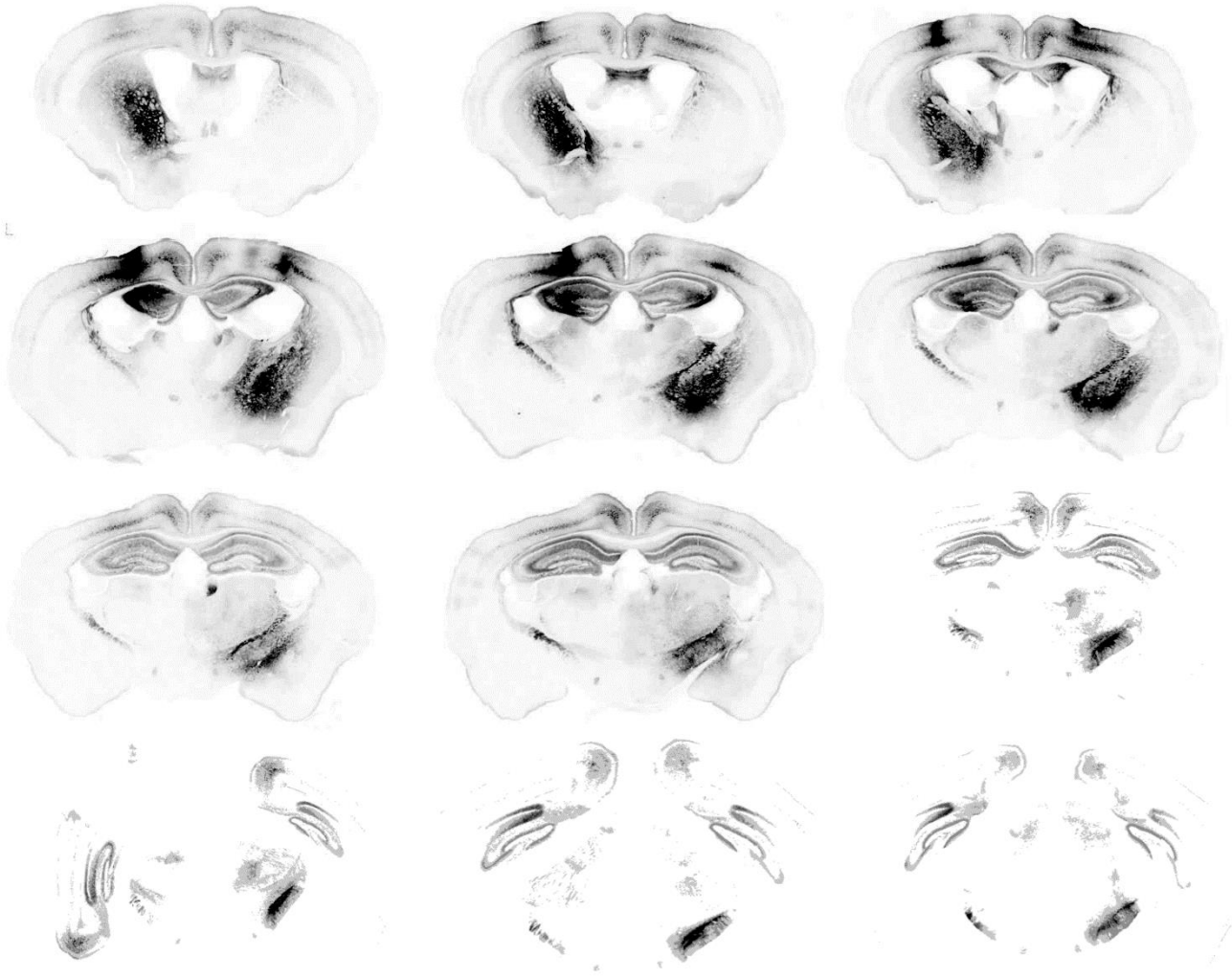


Figure 20. Example of serial sections of an adult (P120) mouse brain showing the viral construct-mediated GFP expression after bilateral intraventricular injection at P2. Bilateral injections in the ventricles at P2 drives the expression of GFP in several regions in the adult brain (hippocampus, cortex, thalamic and hypothamic regions).

Next, we assessed the ability of the previously tested AVV9 vector in driving GFP expression (as a proxy of NKCC1 downregulation) in the adult brain upon bilateral intraventricular injection with the AAV9-NKCC1 amiR and control constructs at P2 in WT and Ts65Dn pups after random allocation of the treatment. Mice were weaned at P28. First, we assessed the transduction efficiency by histochemistry experiments, 15 brains were collected between P120-P130, and processed for immunohistochemistry with the nuclear dye Hoechst-33342 on serial brain slices.

After intraventricular injection at P2, we found that GFP was present in hippocampus, cortex and subcortical areas of all the 15 adult brains we analyzed (Figure 20).

4. Neuron-specific NKCC1 knockdown by AAV9 infection early-in-life rescues memory deficits in adult Ts65Dn mice

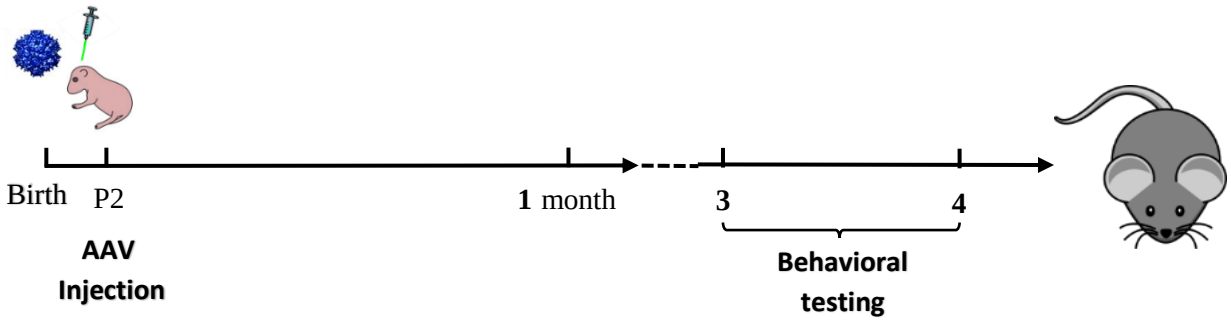


Figure 21. Schematic representation of the experimental protocol for WT and Ts65Dn mice. WT and Ts65Dn pups at postnatal day 2 are subjected to bilateral intraventricular injection of the neuron-specific AAV9 construct for NKCC1 knockdown. Mice are weaned at P28 and behavioral testing is performed between P90-120.

We first assessed the long-term effects of early-in-life knockdown of NKCC1 in restoring short-term memory in adult life, by testing WT and Ts65Dn mice expressing NKCC1 or control amiR in the T-maze test (spontaneous alteration protocol, 11 trials). In agreement with data from the literature (Kleschevnikov, Belichenko et al. 2012, Savardi, Borgogno et al. 2020), control amiR Ts65Dn mice showed poor short-term memory in comparison to control amiR WT mice. NKCC1 amiR rescued the number of correct choices of Ts65Dn mice in comparison to trisomic animals injected with the control construct (Figure 22). WT NKCC1 amiR animals did not show any significant phenotype.

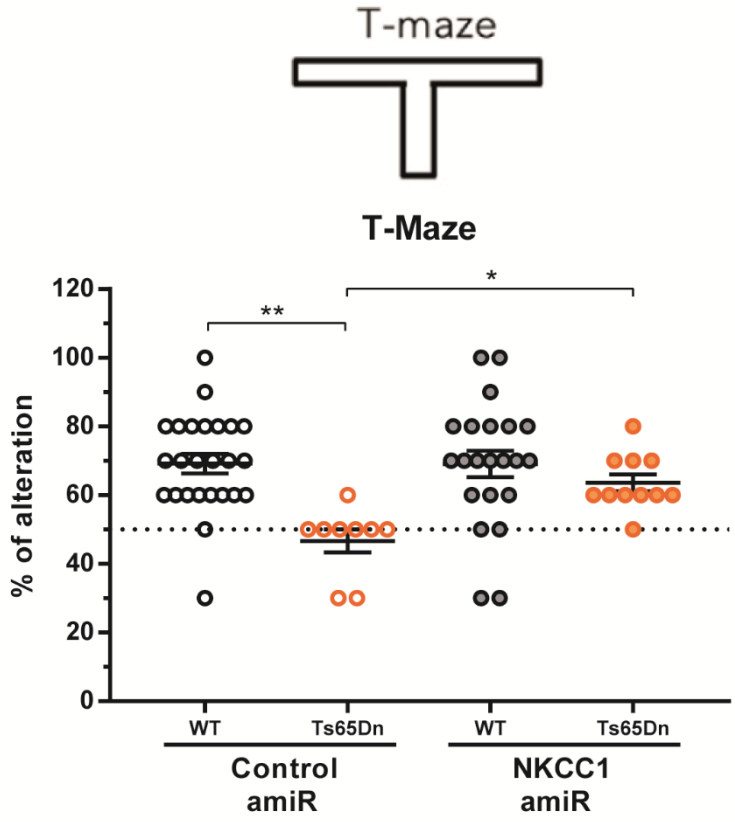


Figure 22. Neuron-specific NKCC1 knockdown by AAV9 infection early-in-life rescues impaired short-term memory in adult Ts65Dn mice. Top, schematic representation of the T-maze task. Bottom, quantification of the correct choices in mice injected with control construct or amiR NKCC1 at P2. Lines report the average score of all analyzed animals (± SEM) and circles represent data points for each animal (two-way ANOVA, $F_{Interaction}(1,63)= 4.835$, $p = 0.0316$, Holm–Sidak post hoc test, ** $p < 0.01$, * $p < 0.05$ two-way).

Next, we assessed any lasting effects of early-in-life RNA interference against NKCC1 on long-term memory deficits in Ts65Dn adult mice. Long-term memory was assessed in three independent tasks (i.e., novel object recognition, contextual fear conditioning and object location). The novel-object recognition (NOR) test measures the preference of mice for a novel object *versus* previously encountered familiar objects. In agreement with what previously demonstrated (Deidda, Parrini et al. 2015), Ts65Dn mice injected at P2 with control amiR showed deficits in recognition memory in comparison to WT control amiR animals. Interestingly, early-in-life NKCC1 downregulation was able to fully rescue the deficit in discrimination of novelty of Ts65Dn mice when compared to control amiR Ts65Dn mice in adulthood (Figure 23A). The effect of NKCC1 amiR in the trisomic group in NOR test was not due to object preference (among the 3 objects used in this

behavioral test) or in total object exploration time in all groups (Figure 23 B,C,D). WT NKCC1 amiR animals did not show any significant phenotype.

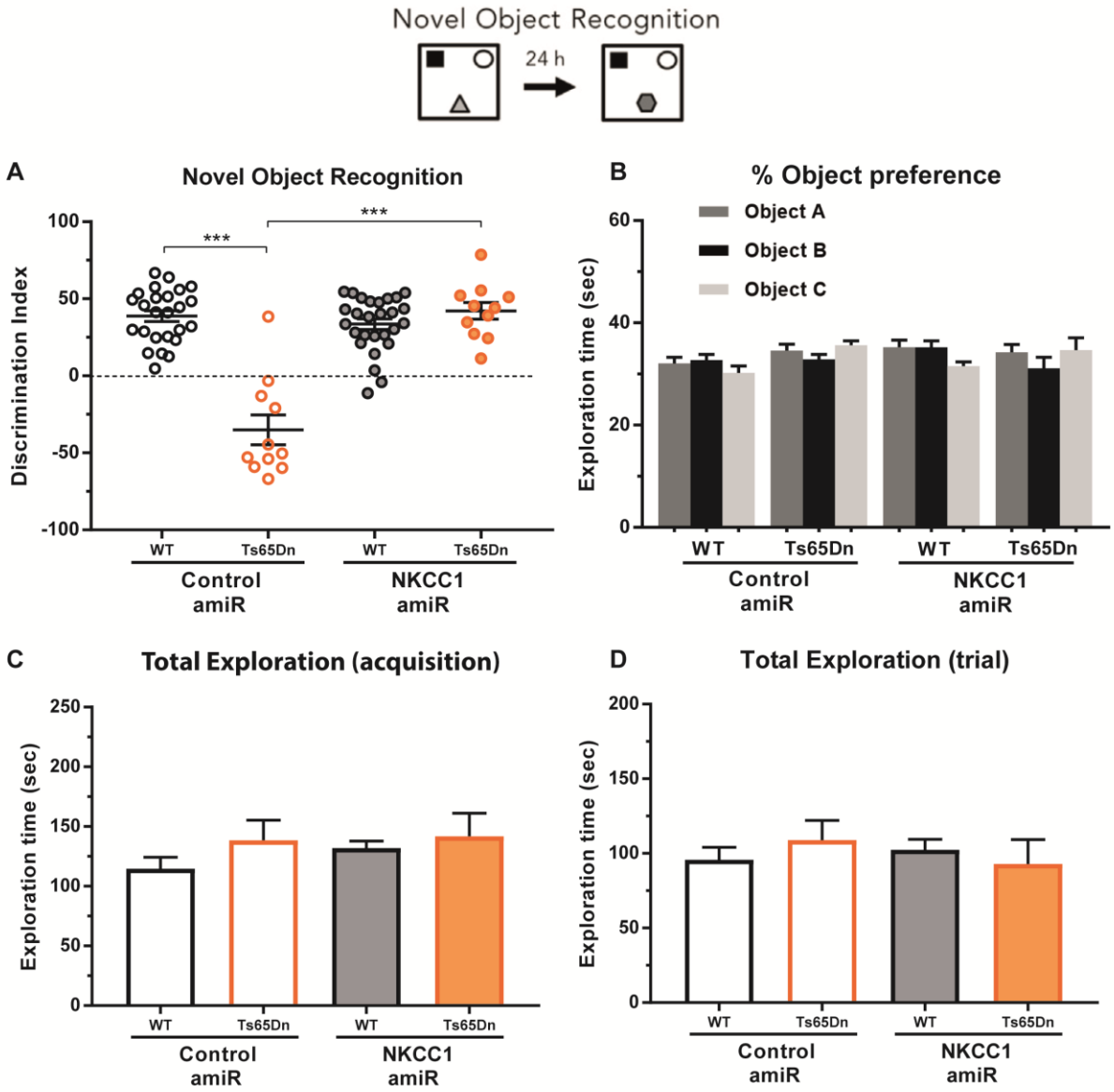


Figure 23. Neuron-specific NKCC1 knockdown by AAV9 infection early-in-life rescues impaired discrimination index in the novel object recognition task in adult Ts65Dn mice. Top, schematic representation of the novel object recognition test. Bottom, **(A)** Quantification of the discrimination index in mice injected with control construct or NKCC1 amiR at P2. Lines report the average discrimination index of all analyzed animals (± SEM) and circles represent data points for each animal (two-way ANOVA, $F_{Interaction}$ (1,70)= 63.78, p = 0.0001, Tukey's post hoc test, *** p < 0.001, two-way). **(B)** Quantification of the percentage of time spent exploring the three objects during the acquisition phase. Bars represent the average exploration time for each object of all analyzed animals ± SEM (ANOVA, *p > 0.05). **(C)** Quantification of the percentage of the total time spent exploring the objects during the acquisition phase.

Bars represent the average exploration time (seconds) of all analyzed animals ± SEM (two-way ANOVA, $F_{Interaction}$ (1,70)= 0.3491, p = 0.5566, Tukey's post hoc test, two-way). **(D)** Quantification of the percentage of the total time spent exploring the objects during the trial phase. Bars represent the average exploration time (seconds) of all analyzed animals ± SEM (two-way ANOVA, $F_{Interaction}$ (1,70)= 1.09, p = 0.3001, Tukey's post hoc test, two-way).

Next, we evaluated associative memory in the contextual fear conditioning test (CFC). This task assesses long-term, associative learning by quantifying the freezing response that takes place after conditioning by the pairing of a foot shock with a particular context represented by the grid releasing the shock (Figure 24, Top). In agreement with previously demonstrated (Deidda, Parrini et al. 2015), control amiR Ts65Dn mice showed poor freezing response after re-exposure to the grid context 24h after the conditioning session, when compared to control amiR WT mice. Notably, NKCC1 amiR intraventricular injection early-in-life fully restored the associative memory in adult Ts65Dn mice (Figure 24A), with no changes in non-associative freezing (freezing response measured in a new context; Figure 24B). WT NKCC1 amiR animals did not show any significant phenotype.

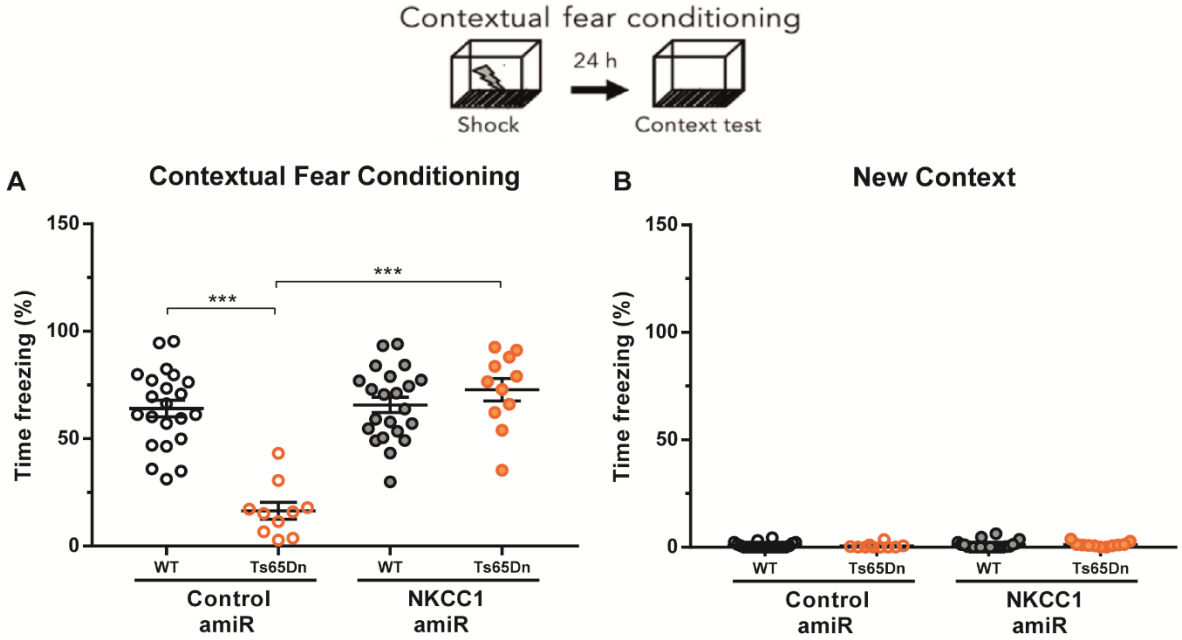


Figure 24. Neuron-specific NKCC1 knockdown by AAV9 infection early-in-life rescues impaired freezing response in the contextual fear conditioning task in adult Ts65Dn mice. Top, schematic representation of the contextual fear conditioning test. Bottom, **(A)** Quantification of the freezing response in mice injected with control construct or NKCC1 amiR at P2. Lines report the average discrimination index of all analyzed animals (± SEM) and circles represent data points for each animal (two-way ANOVA, $F_{Interaction}$ (1,61)= 37.74, p = 0.0001, Tukey's post hoc test, *** p < 0.001, two-way). **(B)** Quantification of

the freezing response in mice during the exposition to the new context. Lines report the average discrimination index of all analyzed animals (± SEM) and circles represent data points for each animal (two-way ANOVA, $F_{Interaction}(1,61)= 0.2137$, $p = 0.6455$, Tukey's post hoc test).

Finally, we evaluated spatial memory in the object location task (OL). The test measures the ability of mice to recognize the new location of a familiar object with respect to spatial external cues (Figure 25, top). In agreement with what previously described (Deidda, Parrini et al. 2015), control amiR Ts65Dn mice showed impaired spatial memory in comparison to control amiR WT mic. This was demonstrated by a poor discrimination index reflective of poor discernment of the new object position. NKCC1 amiR significantly restored the performance of Ts65Dn mice (Figure 25A). The effect of the NKCC1 amiR in the OL test was not due to alterations in the object preference or in the total object exploration during the acquisition phase (Figure 25B and 25C). WT NKCC1 amiR animals did not show any significant phenotype. There were no significant differences among any groups in the total exploration time both in the acquisition and trial sessions.

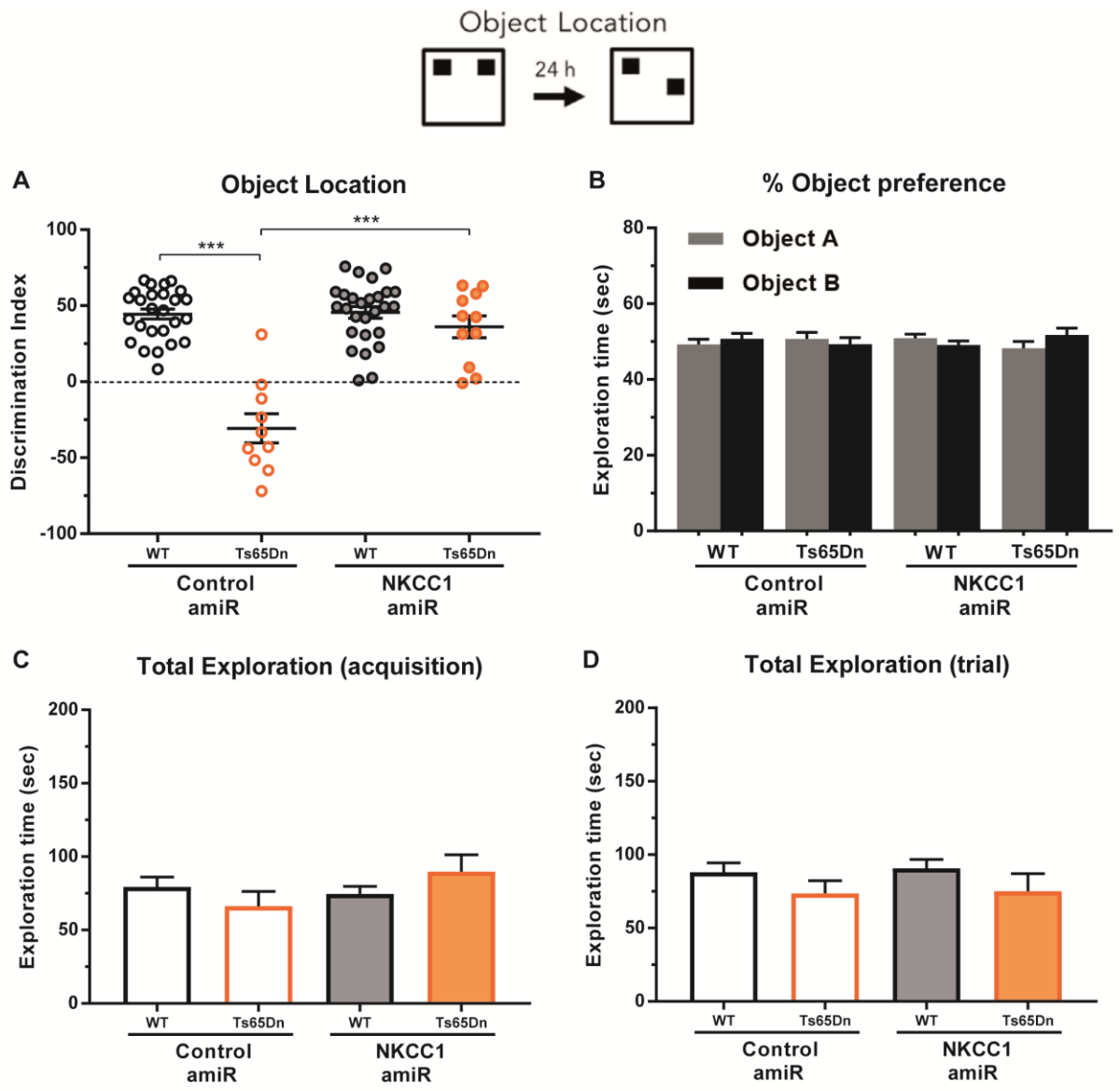


Figure 25. Neuron-specific NKCC1 knockdown by AAV9 infection early-in-life rescues impaired discrimination index in the object location task in adult Ts65Dn mice. Top, schematic representation of the object-location test. Bottom, **(A)** Quantification of the discrimination index in mice injected with control construct or NKCC1 amiR at P2. Lines report the average discrimination index of all analyzed animals (± SEM) and circles represent data points for each animal (two-way ANOVA, $F_{Interaction}$ (1,70)= 36.42, p = 0.0001, Tukey's post hoc test, *** p < 0.001, two-way). **(B)** Quantification of the percentage of time spent exploring the two objects during the acquisition phase. Bars represent the average exploration time for each object of all analyzed animals ± SEM (ANOVA, *p > 0.05). **(C)** Quantification of the percentage of the total time spent exploring the objects during the acquisition phase. Bars represent the average exploration time (seconds) of all analyzed animals ± SEM (two-way ANOVA, $F_{Interaction}$ (1,70)= 3.014, p = 0.0870, Tukey's post hoc test, two-way). **(D)** Quantification of the percentage of the total time spent exploring the objects during the trial phase. Bars represent the average exploration time (seconds) of all analyzed animals ± SEM (two-way ANOVA, $F_{Interaction}$ (1,70)= 0.004126, p = 0.9490, Tukey's post hoc test, two-way).

5. Neuron-specific NKCC1 knockdown by AAV9 injection early-in-life rescues increased susceptibility to epileptic seizures in adult Ts65Dn mice

Deidda and colleagues reported no significant effect of bumetanide on the increased susceptibility to epileptic seizures described in Ts65Dn mice (Westmark, Westmark et al. 2010, Deidda, Parrini et al. 2015), when the drug-treatment was performed in adulthood (Deidda, Parrini et al. 2015). To assess whether early knockdown of NKCC1 could rescue the susceptibility to seizure phenotype of trisomic mice, we performed the audiogenic seizure (AGS) test in adult Ts65Dn and WT mice injected with control and NKCC1 amiR at P2. In agreement with the literature (Deidda, Parrini et al. 2015), Ts65Dn control amiR mice exhibited increased AGS susceptibility in comparison to WT control amiR mice, as quantified by the percentage of animals that exhibited AGS (Figure 26A), or mean AGS severity score (Figure 26B). Notably, NKCC1 amiR significantly restored the increased AGS susceptibility in Ts65Dn mice.

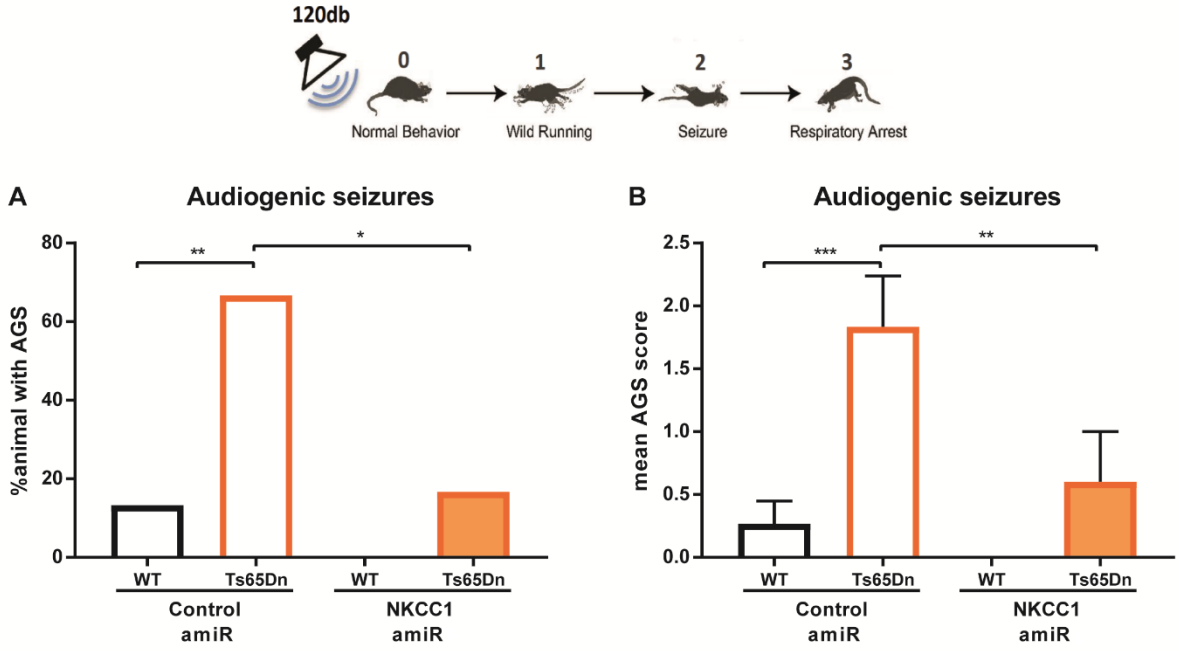
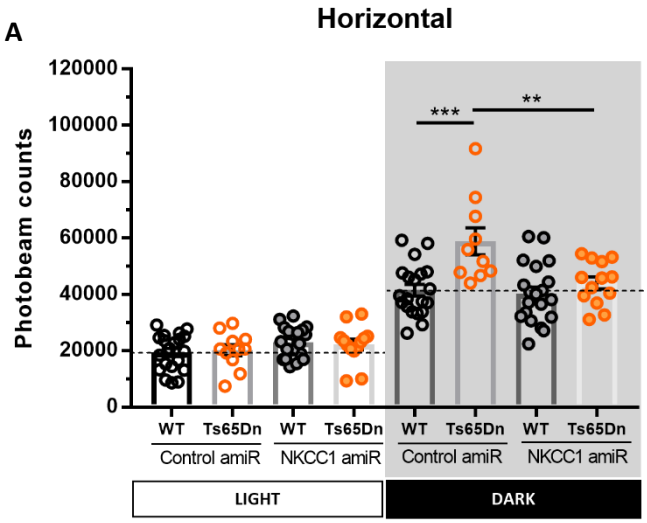


Figure 26. Neuron-specific NKCC1 knockdown by AAV9 infection early-in-life rescues increased susceptibility to seizures in adult Ts65Dn mice. Top, schematic representation of the AGS test. The numbers indicate the score given to an animal during the procedure for seizure induction by a 120 dB white noise. Bottom, **(A)** Percentage of animals that exhibited AGS susceptibility (left, Chi Square test with Sidak adjustment for multiple comparisons, **P<0.01, *P < 0.05), (**B**) mean AGS severity score (ANOVA on ranks, Student-Newman-Keuls Method, (*P < 0.05). Bar charts represent the percentage of animals showing AGS (left) or average severity score ± SEM (right). The numbers of animals tested (WT control amiR: 15, Ts65Dn control amiR: 12, WT NKCC1 amiR: 20, Ts65Dn NKCC1 amiR: 12).

6. Neuron-specific NKCC1 knockdown by AAV9 injection early-in-life rescues increased locomotor activity in adult Ts65Dn mice

Deidda and colleagues reported no significant effect of bumetanide on the increased locomotor activity typical of Ts65Dn animals (Escorihuela, Fernandez-Teruel et al. 1995, Reeves, Irving et al. 1995, Sago, Carlson et al. 2000, Deidda, Parrini et al. 2015), when bumetanide was given in adult trisomic mice. To assess whether early knockdown of NKCC1 could rescue the hyperactivity phenotype of adult trisomic mice, we performed a 24-hour monitoring test of the locomotor activity of adult Ts65Dn and WT mice injected with control and NKCC1 amiR at P2. The 24-hour monitoring was divided in 12 hours of light and 12 hours of dark. As reported in the literature (Deidda, Parrini et al. 2015), Ts65Dn control amiR mice exhibited increased locomotor activity for all the parameters analyzed (horizontal activity, total distance travelled and stereotypic activity) in comparison to WT control amiR mice in the dark phase, when mice are more active. Notably, NKCC1 amiR in Ts65Dn fully restored the increased locomotor activity (horizontal, total distance travelled and stereotypic) (Figure 27A,B,C). No significant effect of NKCC1 amiR was observed in WT animals. On the other hand, no significant differences were found in the locomotor activity between Ts65Dn and WT control amiR groups during the light phase (Figure 27A,B,C), and, early knockdown of NKCC1 with NKCC1 amiR did not have any significant phenotype in both WT and Ts65Dn.



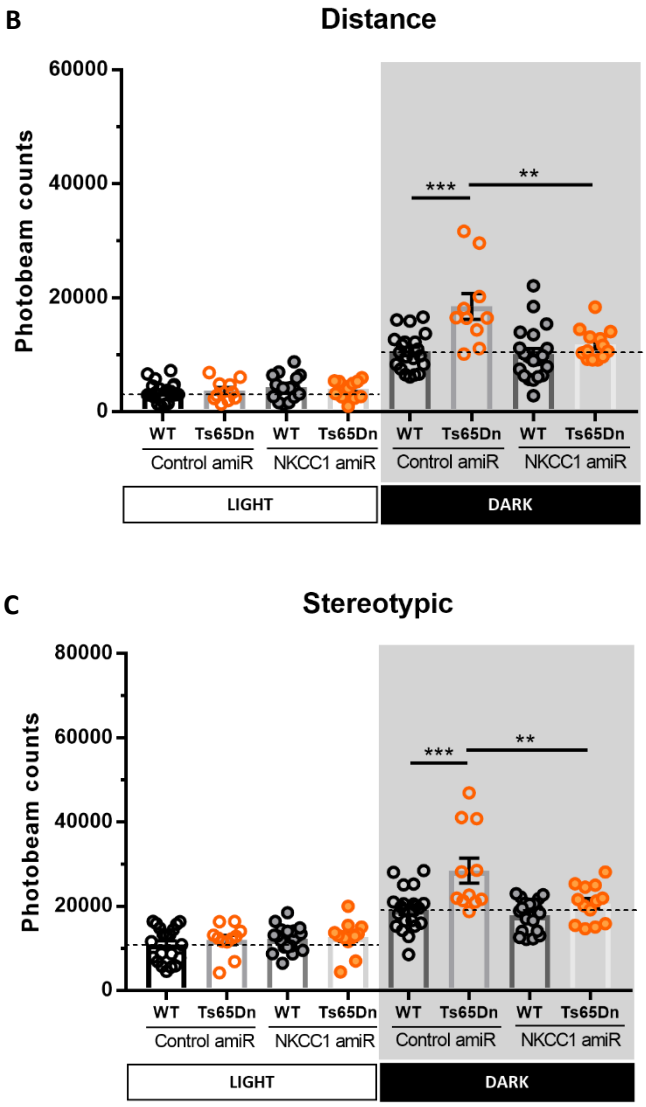


Figure 27. Neuron-specific NKCC1 knockdown by AAV9 infection early-in-life rescues increased locomotor activity in adult Ts65Dn mice. (A) Quantification of the horizontal locomotor of WT and Ts65Dn adult mice injected with either control or NKCC1 amiR at P2. The light phase is represented on the left and whereas the dark phase is represented shadowed on the right. Bars represent the average locomotor activity ± SEM, and circles represent data points for each animal. Light phase: two-way ANOVA, $F_{Interaction}$ (1,59)= 0.2535, p = 0.6165, Tukey's post hoc test, * p < 0.05, two-way. Dark phase: two-way ANOVA, $F_{Interaction}$ (1,59)= 5.843, p = 0.0188, Tukey's post hoc test, * p < 0.05, two-way. **(B)** Quantification of the total distance travelled by adult WT and Ts65Dn mice injected with either control or NKCC1 amiR at P2. The light phase is represented on the left and whereas the dark phase is represented shadowed on the right. Bars represent the average locomotor activity ± SEM, and circles represent data points for each animal. Light phase: two-way ANOVA, $F_{Interaction}$ (1,57)= 0.2665, p = 0.6077, Tukey's post hoc test, * p < 0.05, two-way. Dark phase: two-way ANOVA, $F_{Interaction}$ (1,59)= 6.94, p = 0.0107, Tukey's post hoc test, * p < 0.05, two-way. **(C)** Quantification of the stereotypic locomotor activity of WT and

Ts65Dn adult mice injected with either control or NKCC1 amiR at P2. The light phase is represented on the left and whereas the dark phase is represented shadowed on the right. Bars represent the average locomotor activity ± SEM, and circles represent data points for each animal. Light phase: two-way ANOVA, $F_{Interaction}$ (1,59)= 0.1672, p = 0.6841, Tukey's post hoc test, * p < 0.05, two-way. Dark phase: two-way ANOVA, $F_{Interaction}$ (1,59)= 4.768, p = 0.0330, Tukey's post hoc test, * p < 0.05, two-way.

7. Neuron-specific NKCC1 knockdown by AAV9 infection early-in-life rescues $GABA_A$ergic signaling in hippocampal acute slices of adult Ts65Dn mice

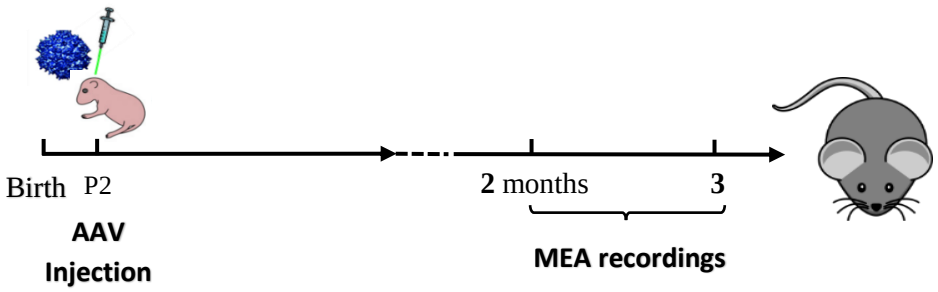


Figure 28. Schematic representation of the experimental protocol for WT and Ts65Dn mice. WT and Ts65Dn pups at postnatal day 2 are subjected to bilateral intraventricular injection of the neuron-specific AAV9 construct for NKCC1 knockdown. Mice are weaned at P28 and electrophysiological recordings are performed between P60-90.

Next, we wanted to investigate the possible mechanisms underlying the behavioral rescue upon NKCC1 downregulation early-in-life in Ts65Dn animals. Regulation of intracellular Cl^- concentration by Cl^- transporter NKCC1 and KCC2 is a key player in modulating GABAergic responses and consequent physiological brain development (Cancedda, Fiumelli et al. 2007, Ben-Ari 2017). Thus, we assessed whether NKCC1 inhibition in the context of the increased NKCC1/KCC2 ratio that we found in Ts65Dn pups, could rescue the brain developmental trajectory of Ts65Dn pups. First, we assessed whether the RNA interference-mediated downregulation of NKCC1 early in brain development could rescue the increased GABA-mediated spiking events already described in adult Ts65Dn mice (Deidda, Parrini et al. 2015). To this aim, we performed bilateral intraventricular injection of AAV9-NKCC1 amiR and control constructs to WT and Ts65Dn pups at P2, and performed multi-electrode array recordings (MEA) in acute brain slices (at >P70) from the previously injected animals. Hippocampal slices were recorded at basal conditions for 30 minutes. In a first set of experiments, we tested the neuronal firing activity upon application of exogenous GABA (100 μM). As expected, both control and

NKCC1 amiR WT slices showed a suppression of the average mean firing rate MFR to reach a values near to zero) (Figure 16A). Interestingly, some slices of NKCC1 amiR WT group increased their MFR upon GABA application, but did not reach a significant difference with respect to WT control amiR (Figure 15A). Conversely, control amiR Ts65Dn slices (Figure 15A) showed significantly increased average MFR ratio upon GABA administration in comparison to WT control amiR slices. Remarkably, NKCC1 amiR significantly restored the response to exogenous GABA bath application in adult Ts65Dn hippocampal slices (Figure 15A).

Then, we quantified the percentage of electrodes for each slices showing significant change in comparison to their baseline level (threshold of 10% change). We found that the majority of the electrodes decreased their firing rate upon activating $GABA_A$Rs with GABA in control amiR WT slices (12% of increasing electrodes, 77 % of decreasing electrodes) or NKCC1 amiR slices (18% of increasing electrodes, 66% of decreasing electrodes) (Figure 15B). Conversely, in control amiR Ts65Dn slices the majority of the active electrodes increased the average MFR level upon exogenous GABA administration (51% of increasing electrodes, 36% of decreasing electrodes) indicating a profound alteration of inhibitory GABAergic signaling in comparison to WT control amiR slices. However, adult Ts65Dn slices from mice injected with NKCC1 amiR at P2 displayed a significant decrease in the MFR with a percentage of MFR variation comparable to WT control amiR (8% of increasing electrodes, 76% of decreasing electrodes), indicating a complete rescue of the inhibitory GABAergic signaling.

Next, we tested the effect of the endogenous GABAergic signaling by bath applying the specific $GABA_A$R antagonist bicuculline (BIC; 20 uM) to NKCC1 and control amiR slices from adult WT and Ts65Dn mice. The average MFR ratio of control amiR WT slices showed a large increase in the firing rate activity upon bicuculline treatment, as expected (Figure 15C). Conversely, control amiR Ts65Dn slices showed a decrease in the average MFR ratio upon bicuculline administration in comparison to control amiR WT slice (Figure 15 C). Remarkably, NKCC1 amiR Ts65Dn slices showed a significant increase in the MFR ratio to levels similar to the control amiR WT slices (Figure 15 C). Interestingly, NKCC1 amiR led to a significant decrease of the MFR in WT slices compared to WT control amiR.

When we quantified the percentage of electrodes for each slices showing significant change in comparison to their baseline level, we found that the majority of the electrodes increased their

firing rate upon blocking $GABA_A$Rs with bicuculline in control amiR WT slices (85% of increasing electrodes, 7% of decreasing electrodes), whereas NKCC1 amiR WT slices showed a significant increase in the MFR compared to control amiR WT slices (53% of increasing electrodes, 35% of decreasing electrodes) (Figure 15D). Conversely, in control amiR Ts65Dn slices the majority of the active electrodes decreased the average MFR level upon blocking $GABA_A$Rs with bicuculline (24% of increasing electrodes, 78% of decreasing electrodes) indicating a profound alteration of inhibitory GABAergic signaling in comparison to WT control amiR slices. However, Ts65Dn slices from adult mice injected with NKCC1 amiR at P2 displayed a significant increase in the MFR with a percentage of MFR variation comparable to WT control amiR slices (95% of increasing electrodes, 1% of decreasing electrodes), indicating a complete rescue of the inhibitory GABAergic signaling.

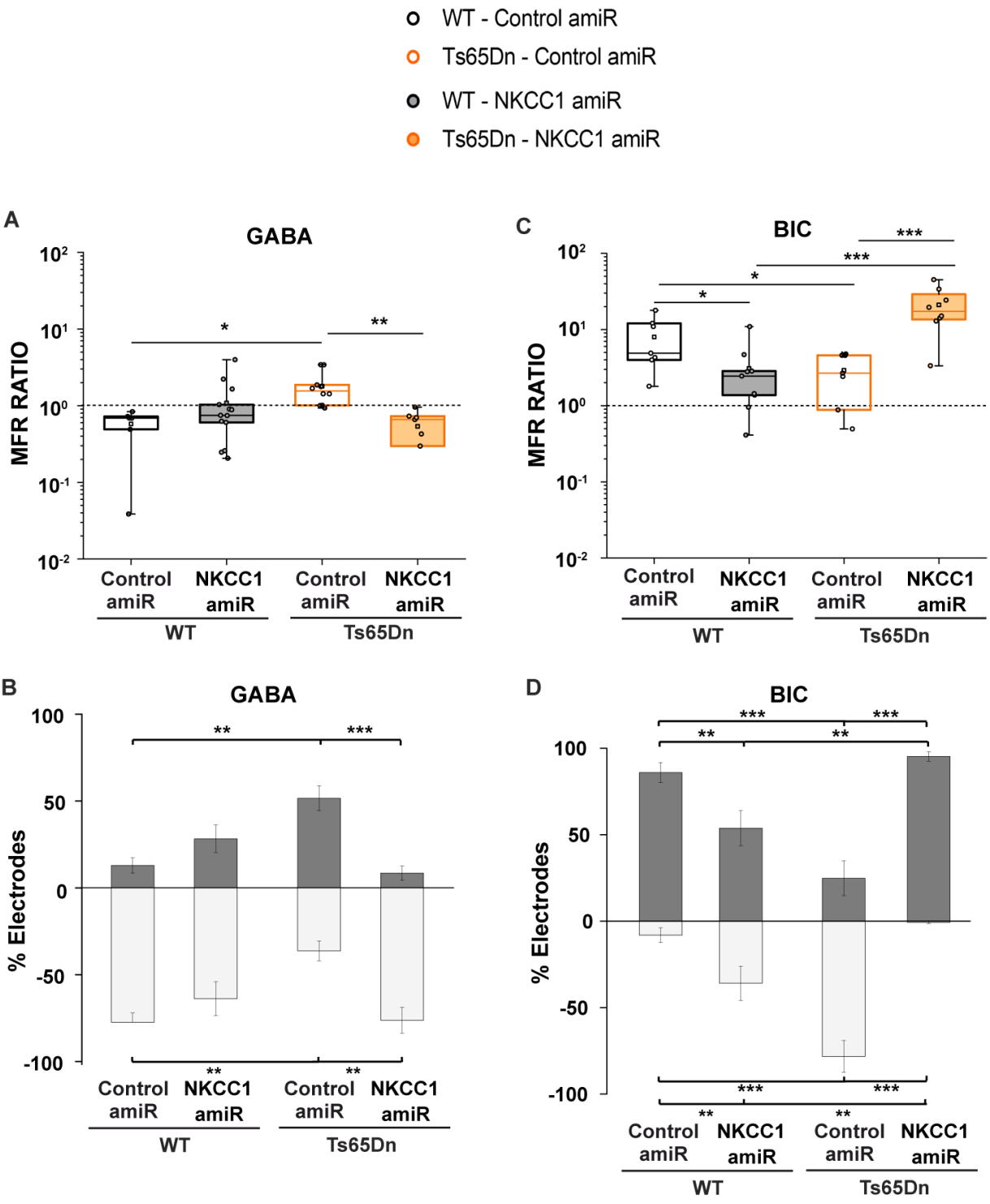


Figure 29. Neuron-specific NKCC1 knockdown by AAV9 infection early-in-life rescues GABAergic signaling in adult Ts65Dn mice. Hippocampal multi-electrode array (MEA) recordings from (8-12 weeks old) adult WT and Ts65Dn mice injected with the NKCC1 or control amiR at postnatal day 2. **(A)** Quantification of MFR ratio after exogenous GABA administration in the bath. MFR for each electrode is normalized to the value of the same electrode during basal condition (indicated as the dotted line) and then

average for each slice. The small square indicates the mean, the central line illustrates the median, and the box limits indicate the 25th and 75th percentiles and each dot represents the MFR ratio for each recorded brain slice. Bars represent the MFR of all recorded electrodes ± SEM, and dots represent data points for each slice (WT control amiR: 6 animals, 6 slices, Ts65Dn control amiR: 9 animals, 10 slices, WT NKCC1 amiR: 9 animals, 13 slices, Ts65Dn NKCC1 amiR: 6 animals, 7 slices). Data points falling on the dashed line (MFR equal to 0) show no change in the MFR data points falling above the dashed line (MFR ratio >1) show an increase in MFR and data falling below the dashed line (MFR ratio <1) show a decrease in MFR after drug administration in the bath (two-way ANOVA, *p<0.05; **p<0.01; followed by Tukey's post hoc test). **(B)** Quantification of the percentage of MEA electrodes for each slice that significantly change the MFR after bath application of GABA. Bars above zero (dark grey) represent percentage of electrodes that increased their activity in comparison to the baseline after GABA application in the bath, whereas bars below zero (light grey) represent percentage of electrodes that decreased their activity in comparison to the baseline (two-way ANOVA, **p<0.01; *** p<0.001; followed by Tukey's post hoc test). **(C)** Quantification of the MFR ratio after bicuculline administration in the bath. MFR for each electrode is normalized to the value of the same electrode during basal condition (indicated as the dotted line) and then average for each slice. The small square indicates the mean, the central line illustrates the median, and the box limits indicate the 25th and 75th percentiles and each dot represents the MFR ratio for each recorded brain slice. Bars represent the MFR of all recorded electrodes ± SEM, and dots represent data points for each slice (WT control amiR: 7 animals, 7 slices, Ts65Dn control amiR: 7 animals, 8 slices, WT NKCC1 amiR: 10 animals, 9 slices, Ts65Dn NKCC1 amiR: 8 animals, 8 slices). Data points falling on the dashed line (MFR equal to 0) show no change in the MFR data points falling above the dashed line (MFR ratio >1) show an increase in MFR and data falling below the dashed line (MFR ratio <1) show a decrease in MFR after drug administration in the bath (two-way ANOVA, *p<0.05; *** p<0.001; followed by Tukey's post hoc test). **(D)** Quantification of the percentage of MEA electrodes for each slice that significantly change the MFR after bath application of bicuculline. Bars above zero (dark grey) represent percentage of electrodes that increased their activity in comparison to the baseline after GABA application in the bath, whereas bars below zero (light grey) represent percentage of electrodes that decreased their activity in comparison to the baseline (two-way ANOVA, **p<0.01; *** p<0.001; followed by Tukey's post hoc test).

8. Early-in-life bumetanide treatment does not cause adverse effects on the body weight and acoustic startle reactivity reflex in the treated mice

NKCC1 amiR delivery by AAV9 infection early-in-life results in NKCC1 knock down throughout life. This experiment does not allow to evaluate whether reduction of NKCC1 activity for a restricted time early in life is sufficient to have long-lasting effects later in life.

To address this question we took a pharmacological approach by the use of the FDA-approved drug bumetanide. Bumetanide is a NKCC1 and NKCC2 inhibitor that has been used as a diuretic for the last 4 decades (Kharod, Kang et al. 2019). We treated WT and Ts65Dn pups twice a day subcutaneously with vehicle (DMSO) or bumetanide (subcutaneous, 0.2 mg/kg) from postnatal day 2 (P2) to postnatal day 15 (P15) (Figure 30). The two injection a day was followed based on the short elimination half-life (no detection in plasma after 2 hours) and the poor capacity of the drug to penetrate the blood brain barrier (Cleary, Sun et al. 2013, Savardi, Borgogno et al. 2020). Bumetanide was dissolved in DMSO in a stock solution of 1 mg/ml. The day of the injection the stock solution was dissolved in saline accordingly and injected in a volume of 20 µl/g (between P2-P8 due to low pup weight and amount injected) and 10 µl/g (between P9-P15 as pups are gaining weight) to have a final concentration of 0.2 mg/kg. We chose this concentration of bumetanide on the basis of previous studies on rodents (Dzhala, Talos et al. 2005, Mazarati, Shin et al. 2009, Brandt, Nozadze et al. 2010, Deidda, Allegra et al. 2015, Deidda, Parrini et al. 2015, Savardi, Borgogno et al. 2020). We chose to perform the bumetanide treatment from postnatal day 2 to postnatal day 15 following previous studies that demonstrated the safety of bumetanide treatment during this period (Wang and Kriegstein 2011, Marguet, Le-Schulte et al. 2015, He, Arroyo et al. 2019). On bumetanide-treated animals and their littermates controls, we assessed long-term effects of early-in-life treatment in a battery of behavioral tests performed between P90-120 (in absence of treatment).

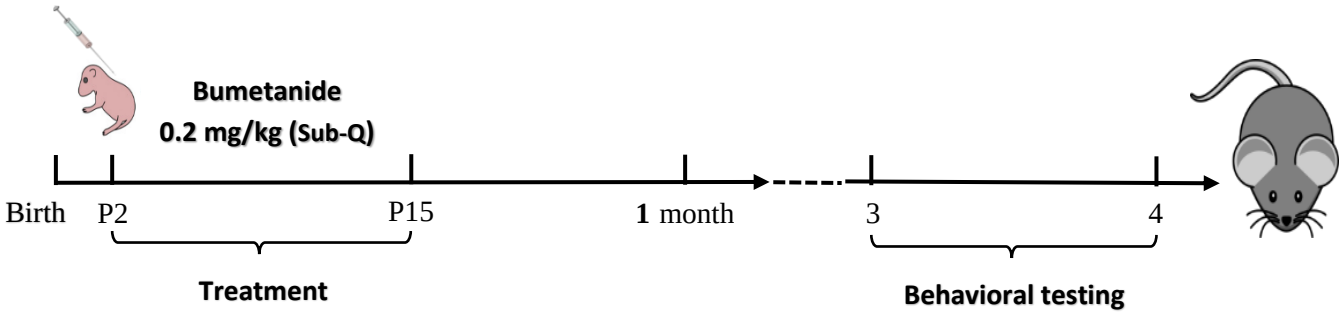
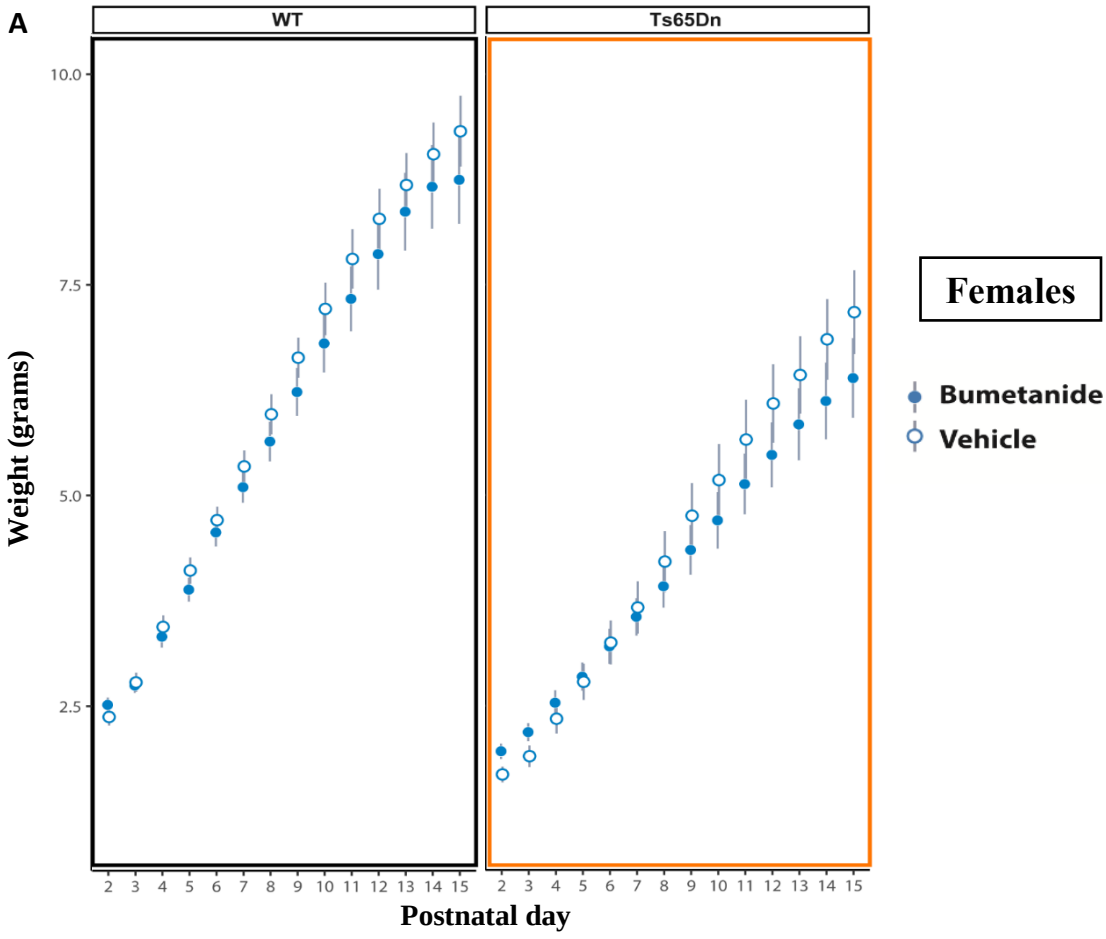


Figure 30. Schematic representation of the experimental protocol for the *in vivo* experiments. Subcutaneous bumetanide injections (0.2 mg/kg) were performed twice a day from postnatal 2 to postnatal day 15 in WT and Ts65Dn pups. Behavioral testing was performed between P90-120 (see Figures 33-44) and tissues collected at the end of the experiment.

Due to the potent diuretic effect of bumetanide that leads to excessive diuresis and possibly hypokalemia we first assessed the safety of the early-in-life treatment in mice. First, we investigated any effects of bumetanide on the body weight of pups during the temporal window of the treatment. We monitored the body weight of the animals treated daily from the first day of treatment (P2) until the last day of the treatment (P15). We found no effect of early bumetanide treatment on body weight of both females and males of WT and Ts65Dn animals when compared to vehicle-treated controls (Figure 31). Of note, we found a significantly higher body weight in WT pups compared to Ts65Dn, in agreement, with data from the literature (Glass, Valmadrid et al. 2019).



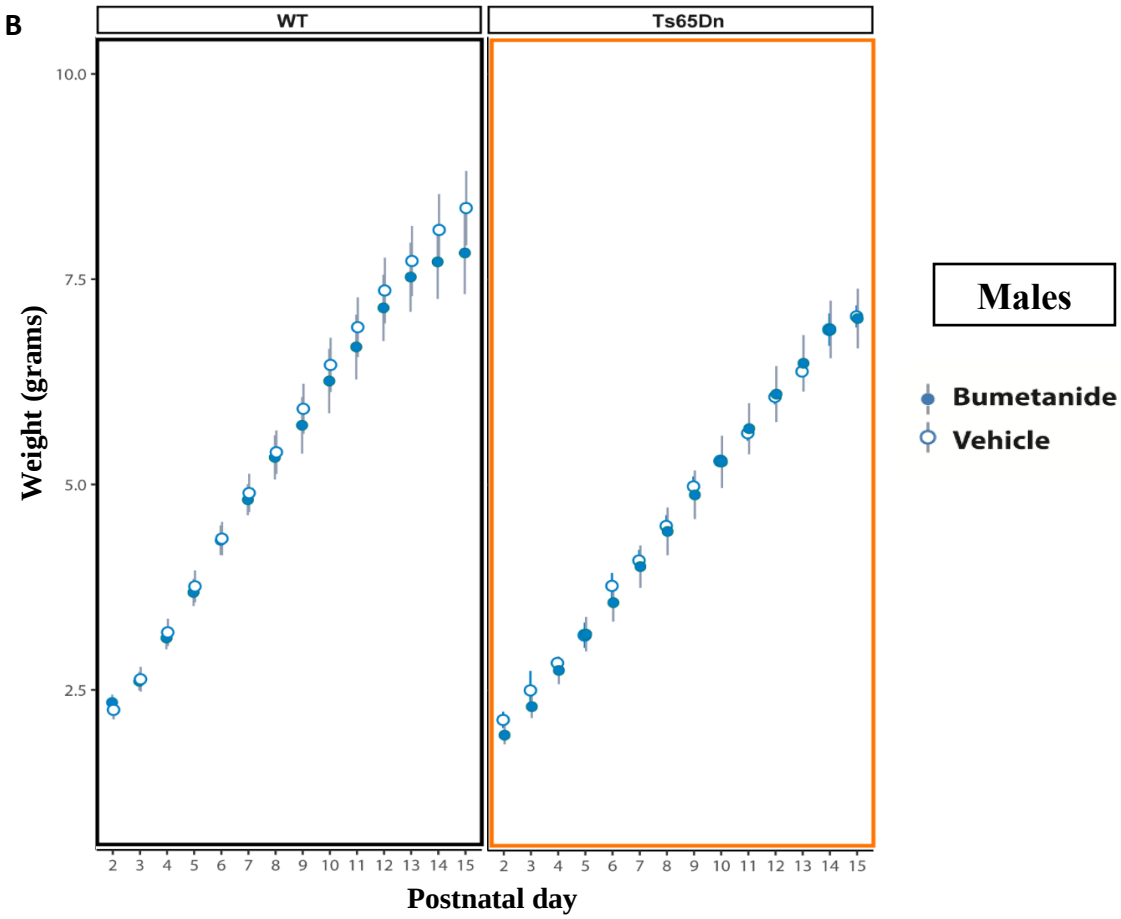


Figure 31. Early-in-life bumetanide treatment does not affect body weight in pups. (A) Average weight of female Ts65Dn and WT animals treated with vehicle or bumetanide in early life, (ANOVA mixed linear models, * $p < 0.05$). Circles report the average weight of all animals (± SEM). **(B)** Mean weight of male Ts65Dn and WT animals treated with vehicle and bumetanide. (ANOVA mixed linear models, * $p < 0.05$). Circles report the average weight of all analyzed animals (± SEM).

Then, since bumetanide treatment in newborn babies has been linked to hearing loss in humans (Pressler, Boylan et al. 2015). We treated pups with vehicle and bumetanide during the first two weeks of life and tested them in the acoustic startle reactivity response test in the adulthood (P90). In particular, we induced a startle response in mice by exposing them to a 90 dB sudden sound. The entity of startle response is considered indicative of the hearing ability. We found no significant effect of early-in-life bumetanide treatment in both WT and Ts65Dn mice (Figure 32).

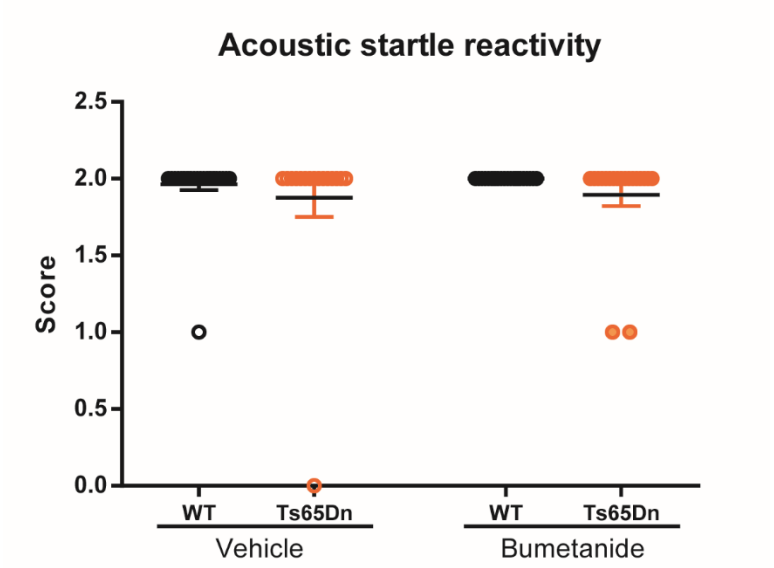


Figure 32. Early-in-life bumetanide treatment does not have an adverse effect on hearing ability in adult Ts65Dn mice. Average score of acoustic startle reactivity response reflex in adult mice treated with vehicle or bumetanide from P2 to P15, (two-way ANOVA, $F_{Interaction}(1,82)=0.2097$, $p = 0.6482$, Tukey's post hoc test). The score scale (0=no response, 1=mild response, 2= complete response) to the acoustic stimulus. Lines report the average startle response of all analyzed animals (± SEM) and circles represent data points for each animal. The numbers of animals tested (WT vehicle: 27, Ts65Dn vehicle: 16, WT bumetanide: 24, Ts65Dn bumetanide: 19).

9. Early-in-life bumetanide treatment rescues long-term memory deficits in adult Ts65Dn mice

First, we assessed the long-term effects of early-in-life bumetanide treatment in restoring short-term memory in adulthood, by testing mice in the T-maze test (spontaneous alteration protocol, 11 trials). Contrary to data from the literature (Savardi, Borgogno et al. 2020), vehicle-treated Ts65Dn mice showed only a non-significant trend toward poorer short-term memory in comparison to vehicle-treated WT mice which was still present in animals treated in early life with bumetanide (Figure 33).

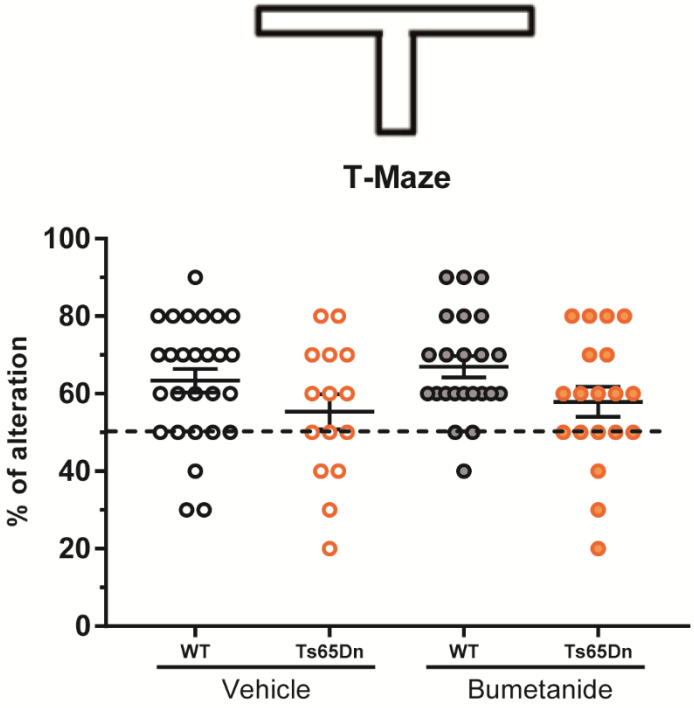


Figure 33. Vehicle-treated Ts65Dn mice do not exhibit a significantly impaired short-term memory and bumetanide treatment does not have any significant effect. Top, schematic representation of the T-maze task. Bottom, quantification of the correct choices in mice treated with vehicle or bumetanide from P2 until P15. Lines report the average score of all analyzed animals (± SEM) and circles represent data points for each animal (two-way ANOVA, $F_{Treatment}(1,80)= 0.7744$, $p = 0.3815$, Tukey's post hoc test, two-way).

In our experiments we included both male and female pups, whereas, the data from the literature included only males (Kleschevnikov, Belichenko et al. 2012, Deidda, Parrini et al. 2015, Savardi, Borgogno et al. 2020). Thus, we decided to analyze males and females separately to assess any gender difference in the performance of our cohort of animals. We found that female Ts65Dn mice did not show poor short-term memory in the T-maze test compared to vehicle-treated female WT (Figure 34A). Bumetanide treatment in early life had no effect in these animals. We found that male Ts65Dn treated with vehicle show a clear trend for poor short-term memory compared to the WT vehicle-treated mice (Figure 34B), which was nevertheless not rescued by bumetanide treatment. To better understand whether there was a gender difference in the performance of the T-maze test in adult Ts65Dn mice, we repeated the experiments in naïve animals (not treated with vehicle early-in-life). We found that adult naïve male Ts65Dn mice showed significantly poorer

short-term memory compared to male WT male animals. Interestingly, adult naïve female Ts65Dn mice did not show any impairment in short-term memory.

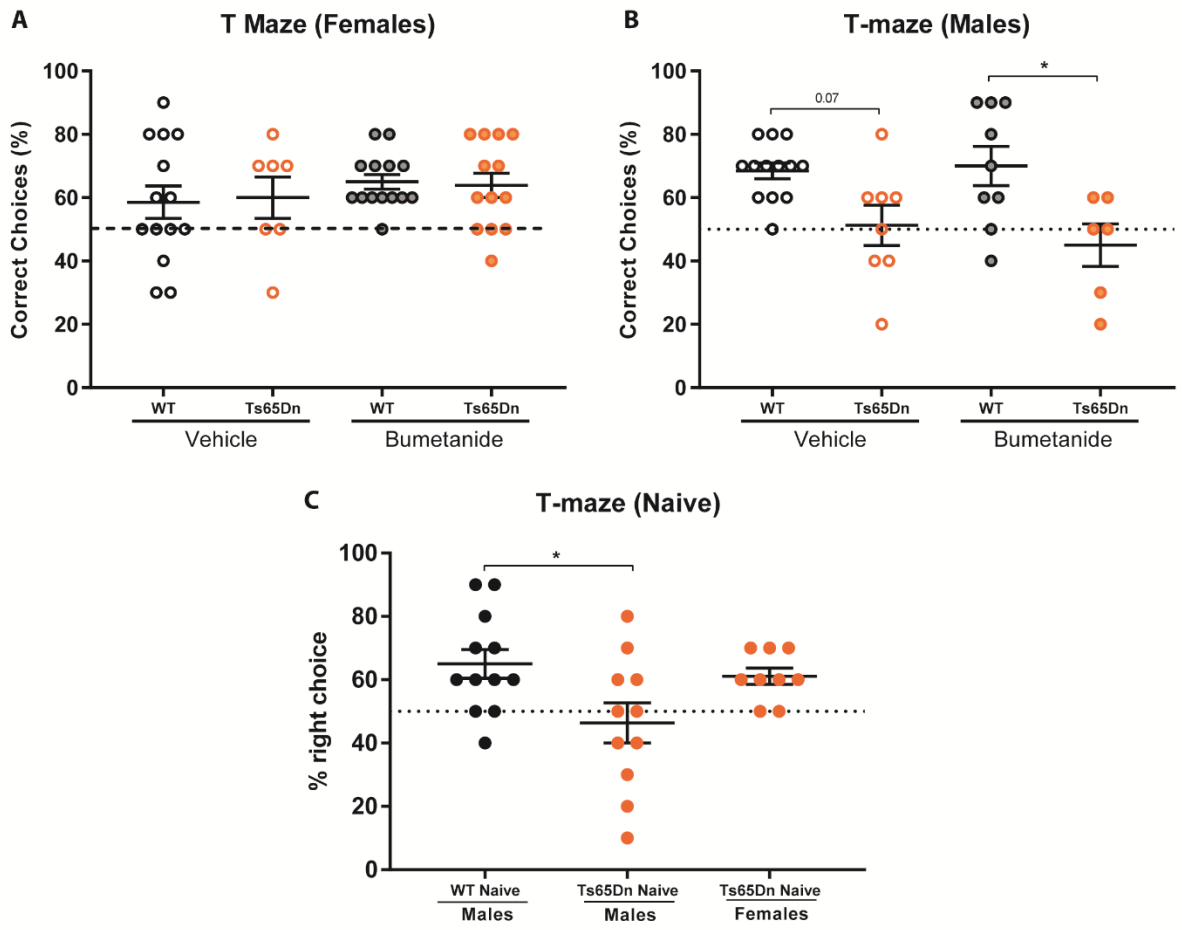


Figure 34. Gender-selective impairment of short-term memory in Ts65Dn mice. (A) Quantification of the correct choices in female WT and Ts65Dn mice injected with vehicle or bumetanide as pups. Lines report the average score of all analyzed animals (± SEM) and circles represent data points for each animal (two-way ANOVA, $F_{Interaction}$ (1,44)= 0.08232, p = 0.7755, Tukey's post hoc test, two-way). **(B)** Quantification of the correct choices in male WT and Ts65Dn mice injected with vehicle or bumetanide as pups. Lines report the average score of all analyzed animals (± SEM) and circles represent data points for each animal (two-way ANOVA, $F_{Interaction}$ (1,32)= 0.5463, p = 0.4652, Tukey's post hoc test, two-way). **(C)** Quantification of the correct choices in naïve male WT, and naïve male and female Ts65Dn mice. Lines report the average score of all analyzed animals (± SEM) and circles represent data points for each animal (Unpaired two-tailed Student's t test (*p < 0.05), p = 0.0247 (naïve WT males- naïve Ts65Dn males; p = 0.5042 (naïve WT males- naïve Ts65Dn females).

Next, we assessed any long-term effects of early-in-life bumetanide treatment on long-term memory deficits in Ts65Dn adult mice. We first performed the novel-object recognition (NOR) test. In agreement with what previously demonstrated and our data in mice injected with control amiR AAV9 viruses (Deidda, Parrini et al. 2015), vehicle-treated Ts65Dn mice showed deficits in recognition memory in comparison to vehicle-treated WT animals. Early-in-life bumetanide treatment was able to fully rescue the deficit in discrimination of novelty of Ts65Dn mice in adulthood in comparison to vehicle-treated WT mice (Figure 35A). The effect of early bumetanide treatment in the trisomic group in NOR test was not due to object preference (among the 3 objects used in this behavioral test) or in total object exploration time in all groups (Figure 35B,C,D). WT animals treated with bumetanide as pups did not show any significant phenotype, in agreement with our results in WT animals infected with NKCC1 amiR in early life.

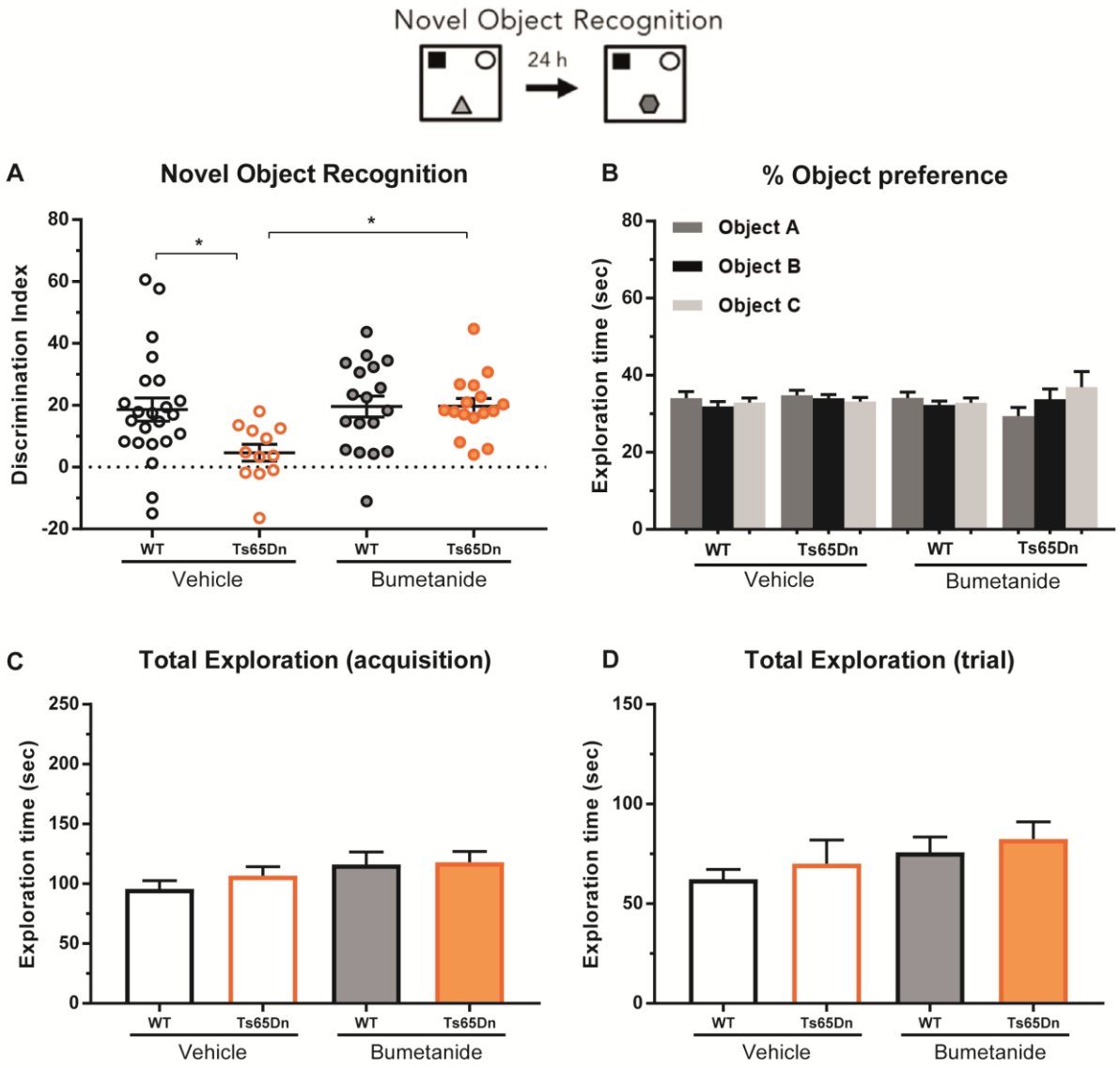


Figure 35. Early-in-life bumetanide treatment rescues impaired long-term memory in the novel object recognition task in adult Ts65Dn mice. Top, schematic representation of the novel object recognition test. Bottom, **(A)** Quantification of the discrimination index in mice treated with vehicle or bumetanide from P2 to P15. Lines report the average discrimination index of all analyzed animals (± SEM) and circles represent data points for each animal (two-way ANOVA, $F_{Interaction}$ (1,65)= 4.015, p = 0.0493, Tukey's post hoc test, * $p < 0.05$, two-way). **(B)** Quantification of the percentage of time spent exploring the three objects during the acquisition phase. Bars represent the average discrimination index of all analyzed animals ± SEM (ANOVA, *$p < 0.05$). **(C)** Quantification of the percentage of the total time spent exploring the objects during the acquisition phase. Bars represent the average exploration time (seconds) of all analyzed animals ± SEM (two-way ANOVA, $F_{Interaction}$ (1,65)= 0.2698, p = 0.6052, Tukey's post hoc test, two-way). **(D)** Quantification of the percentage of the total time spent exploring the objects during the trial phase. Bars represent the average exploration time (seconds) of all analyzed animals ± SEM (two-way ANOVA, $F_{Interaction}$ (1,65)= 0.005437, p = 0.9414, Tukey's post hoc test, two-way).

We found no gender-selective difference in the performance of Ts65Dn mice in the novel object recognition (Figure 36). Both males and females trisomic animals showed a clear trend for impaired recognition memory. In female Ts65Dn mice, bumetanide treatment significantly rescued long-term memory, while in male trisomic animals treated with bumetanide there was a clear trend for restored memory performance, although the number of experimental animals was lower. No significant phenotype was found in WT animals treated with bumetanide both males and females.

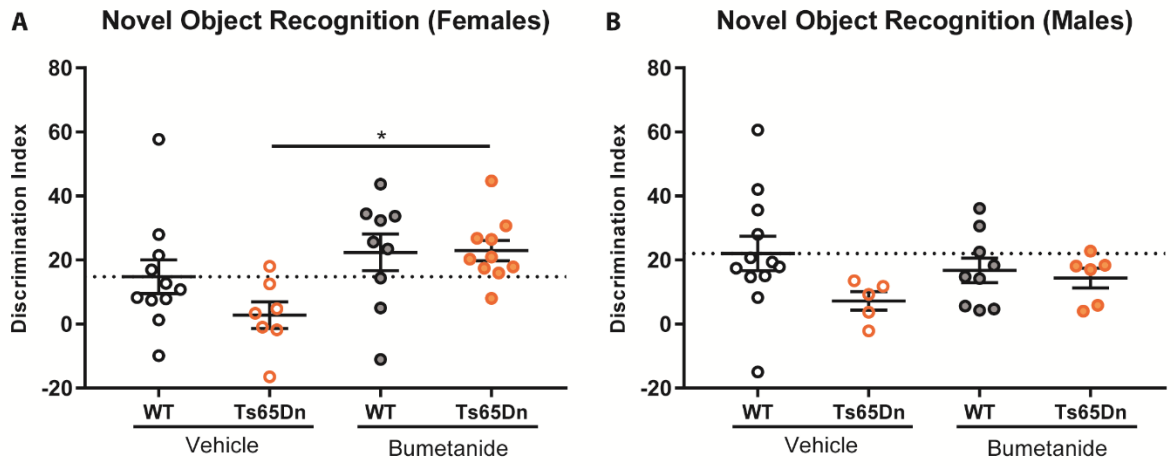


Figure 36. No gender-specific difference in the novel object recognition task. (A) Quantification of the discrimination index in female mice treated with vehicle or bumetanide from P2 to P15. Lines report the average discrimination index of all analyzed animals (± SEM) and circles represent data points for each animal (two-way ANOVA, $F_{Interaction}$ (1,33)= 1.661, p = 0.2064, Tukey's post hoc test, * $p < 0.05$, two-way). **(B)** Quantification of the discrimination index in male mice treated with vehicle or bumetanide from P2 to P15. Lines report the average discrimination index of all analyzed animals (± SEM) and circles represent data points for each animal (two-way ANOVA, $F_{Interaction}$ (1,28)= 1.45, p = 0.2386, Tukey's post hoc test, * $p < 0.05$, two-way).

Next, we evaluated associative memory in the contextual fear conditioning test (CFC). In agreement with previously demonstrated (Deidda, Parrini et al. 2015) and our result in control amiR animals, vehicle-treated Ts65Dn mice showed poor freezing response after re-exposure to the grid context 24h after the conditioning session, when compared to vehicle-treated WT mice. Notably, bumetanide treatment during early postnatal life fully restored the associative memory in

adult Ts65Dn mice (Figure 37A), with no changes in non-associative freezing (freezing response measured in a new context; Figure 37B). WT animals treated with bumetanide did not show any significant phenotype.

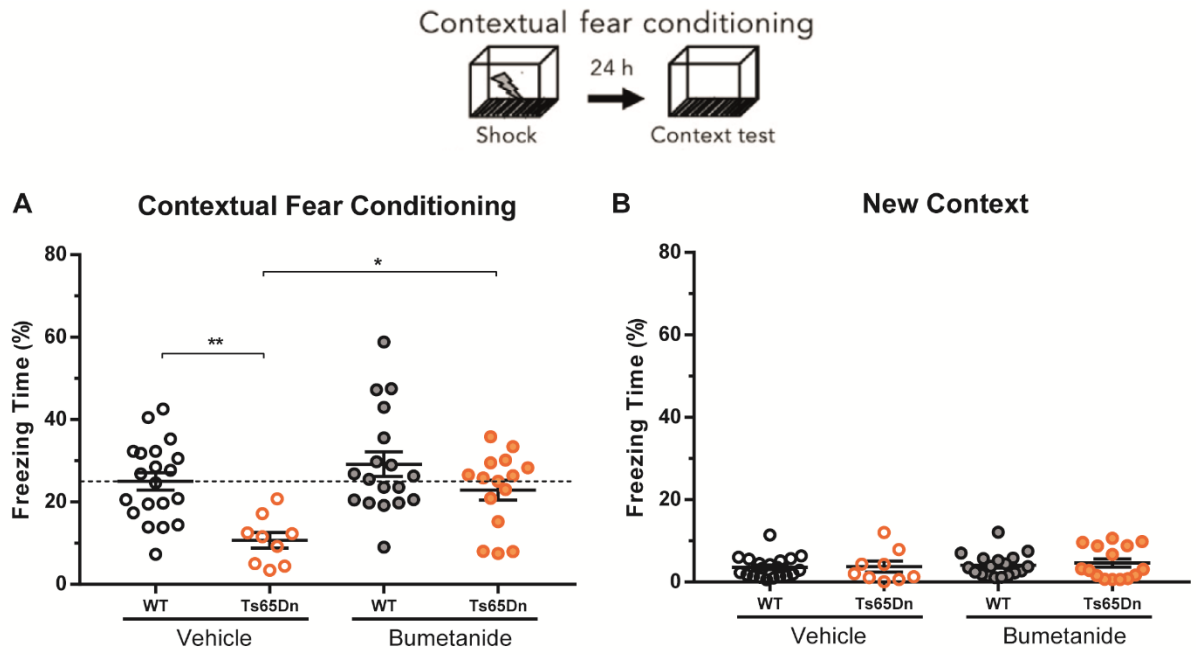


Figure 37. Early-in-life bumetanide treatment rescues impaired freezing response in the contextual fear conditioning task in adult Ts65Dn mice. Top, schematic representation of the contextual fear conditioning test. Bottom, **(A)** Quantification of the freezing response in adult mice treated with vehicle or bumetanide from P2 to P15. Lines report the average freezing response of all analyzed animals (± SEM) and circles represent data points for each animal (two-way ANOVA, $F_{Treatment}$ (1,58)= 9.396, p = 0.0033, Tukey's post hoc test, ** p < 0.01; * p < 0.05, two-way). **(B)** Quantification of the freezing response in mice during the exposition to the new context. Lines report the average freezing response of all analyzed animals (± SEM) and circles represent data points for each animal (two-way ANOVA, $F_{Interaction}$ (1,58)= 0.6462, p = 0.4248, Tukey's post hoc test, two-way).

As for the novel object recognition test, we found no gender-selective difference in the performance of Ts65Dn mice in the contextual fear conditioning (Figure 38). Female trisomic animals showed a clear trend for impaired freezing time, while males showed a significantly impaired freezing response. For both genders, bumetanide treatment improved freezing time in Ts65Dn mice. In agreement with our data on WT animals injected with NKCC1 amiR in early life,

no significant phenotype was found in WT animals treated with bumetanide both in males and females.

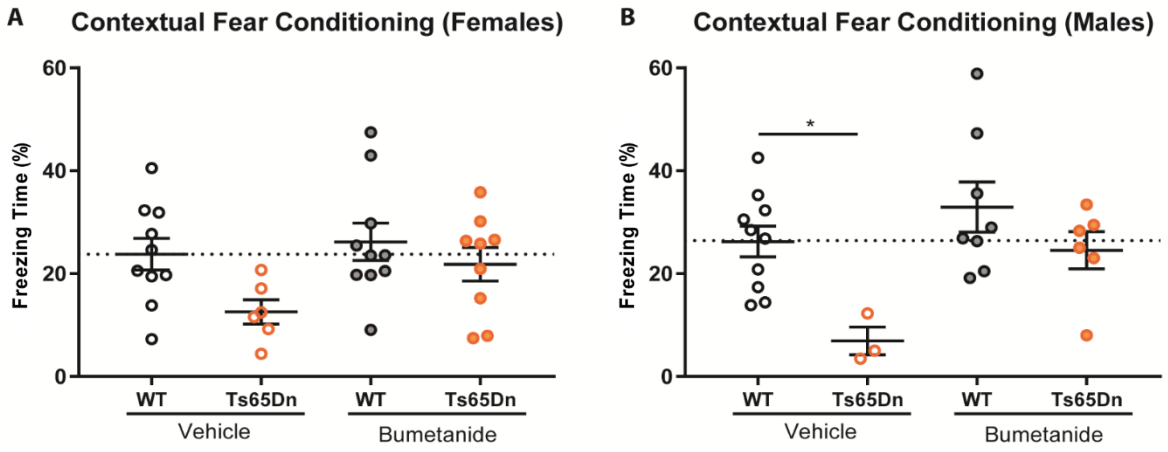


Figure 38. No sex-specific difference in the contextual fear conditioning task. (A) Quantification of the freezing response in adult female mice treated with vehicle or bumetanide from P2 to P15. Lines report the average freezing response of all analyzed animals (± SEM) and circles represent data points for each animal (two-way ANOVA, $F_{Treatment}$ (1,31)= 2.972, p = 0.0947, Tukey's post hoc test, * p < 0.05, two-way). **(B)** Quantification of the freezing response in adult male mice treated with vehicle or bumetanide from P2 to P15. Lines report the average freezing response of all analyzed animals (± SEM) and circles represent data points for each animal (two-way ANOVA, $F_{Treatment}$ (1,23)= 7.302, p = 0.0127, Tukey's post hoc test, ** p < 0.01; * p < 0.05, two-way).

Finally, we evaluated spatial memory in the object location task (OL). In agreement with the literature we found that Ts65Dn mice treated with vehicle show impaired spatial memory as expected (Deidda, Parrini et al. 2015). On the other hand, also WT animals treated with vehicle as pups performed poorly in the OL with a discrimination index very close on average to the chance level (Figure 39A), while early-in-life bumetanide treatment did not show any significant phenotype in Ts65Dn mice and WT mice when compared to their control mice treated with vehicle (Figure 39A). The result of discrimination index in the OL test was not due to alterations in the object preference or in the total object exploration during the acquisition phase (Figure 39B,C). There were no significant differences among any groups in the total exploration time both in the acquisition and trial sessions.

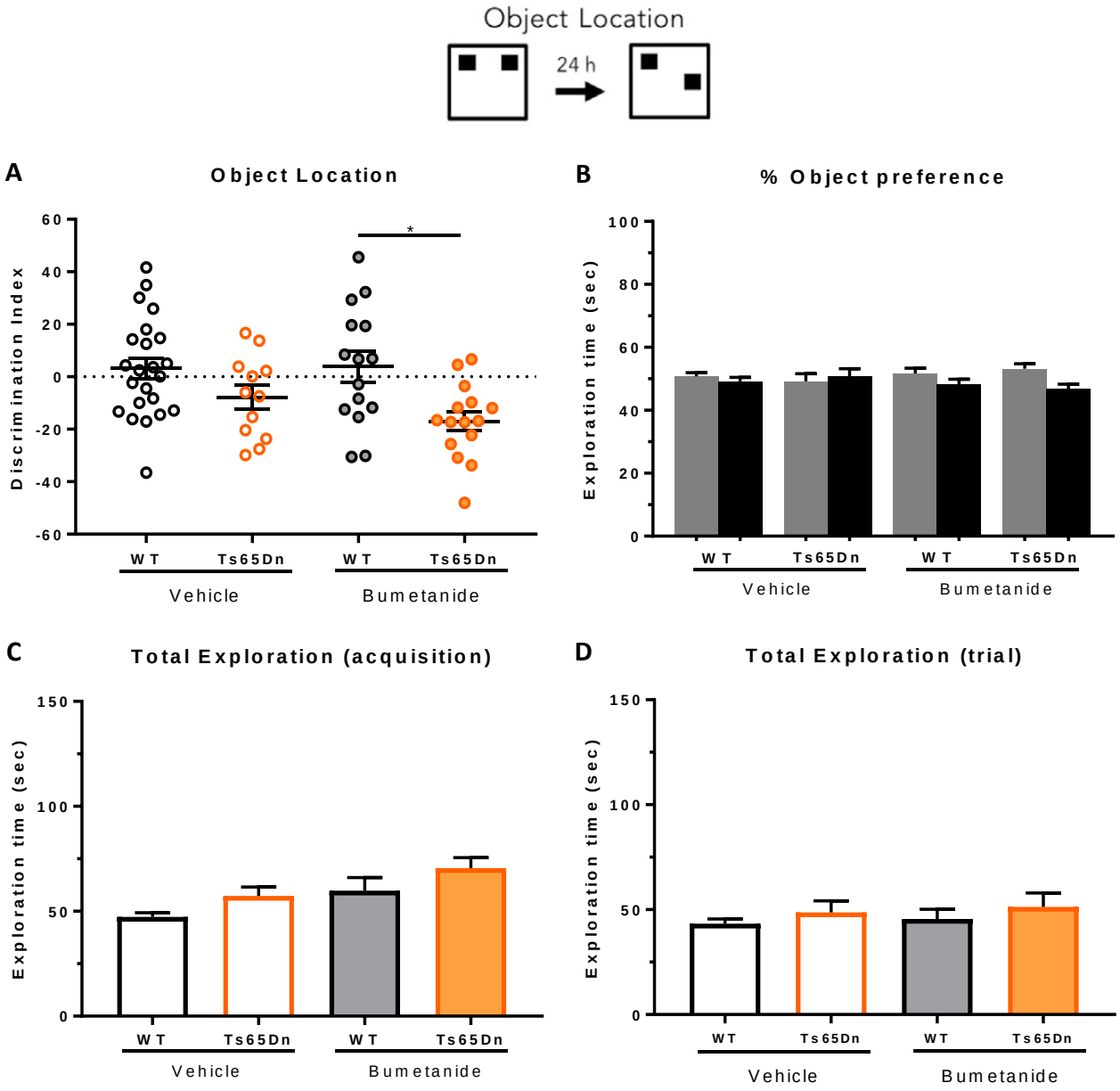


Figure 39. Adult WT vehicle-treated animals as pups show impairment in the object location test. (A) Quantification of the discrimination index in mice treated with vehicle or bumetanide from P2 to P15. Lines report the average discrimination index of all analyzed animals (± SEM) and circles represent data points for each animal (two-way ANOVA, $F_{Interaction}$ (1,61)= 1.097, p = 0.2990, Tukey's post hoc test, * $p < 0.05$, two-way). **(B)** Quantification of the percentage of time spent exploring the two objects during the acquisition phase. Bars represent the average discrimination index of all analyzed animals ± SEM (ANOVA, *$p < 0.05$). **(C)** Quantification of the percentage of the total time spent exploring the objects during the acquisition phase. Bars represent the average exploration time (seconds) of all analyzed animals ± SEM (two-way ANOVA, $F_{Interaction}$ (1,60)= 0.00691, p = 0.9340, Tukey's post hoc test, two-way). **(D)** Quantification of the percentage of the total time spent exploring the objects during the trial phase. Bars represent the average exploration time (seconds) of all analyzed animals ± SEM (two-way ANOVA, $F_{Interaction}$ (1,61)= 0.001591, p = 0.9683, Tukey's post hoc test, two-way).

Data from the literature indicate that early life stress has long-lasting, a gender-selective effect on the performance of male mice in the novel object location task during adulthood (Bath, Nitenson et al. 2017). This effect is specific to the NOL and is not present when animals are tested in the novel object recognition and contextual fear conditioning tests.

We reasoned that injections twice a day during the first two weeks of life could be a stressful event for mouse pups. We thus re-analyzed that data from the OL test taking into account the gender of the animals (Figure 40). We found that female WT animals treated with vehicle were successful in discriminating the novel object location, while female Ts65Dn animals treated with vehicle showed a clear trend for poor spatial memory. Notably, bumetanide treatment in female Ts65Dn animals did not rescue the poor discrimination index compared to the female WT animals treated with bumetanide. On the other hand, male WT animals treated with vehicle showed impaired spatial memory performance in agreement with the poor performance in the OL and Morris water maze (MWM) tests of male animals that have experienced stressful events early-in-life. We found no significant phenotype in male WT and Ts65Dn animals treated with bumetanide.

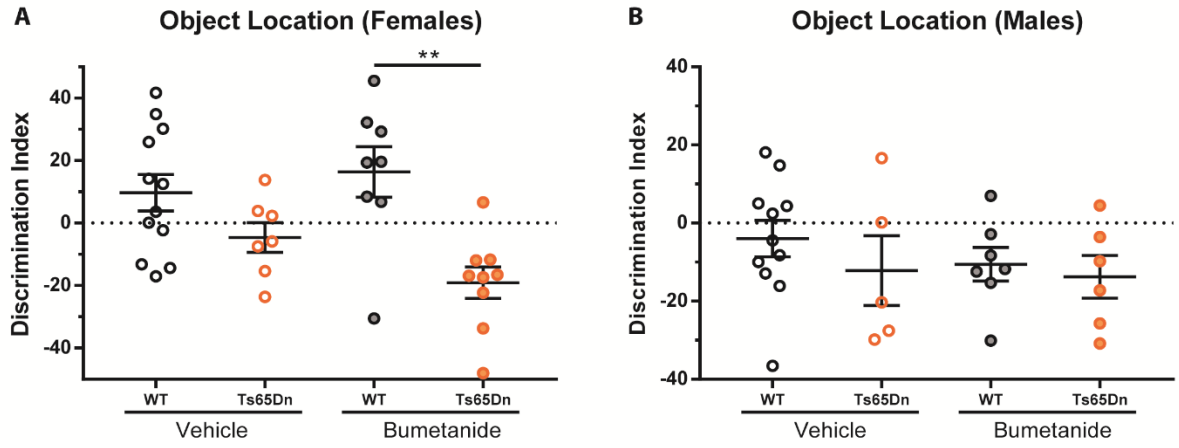


Figure 40. Gender-selective impairment of spatial memory in Ts65Dn mice. (A) Quantification of the discrimination index in adult female mice treated with vehicle or bumetanide from P2 to P15. Lines report the average discrimination index of all analyzed animals (± SEM) and circles represent data points for each animal (two-way ANOVA, $F_{Interaction}(1,32)= 2.833$, $p = 0.1021$, Tukey's post hoc test, * $p < 0.05$, two-way). **(B)** Quantification of the discrimination index in adult male mice treated with vehicle or bumetanide from P2 to P15. Bars represent the average discrimination index of all analyzed animals ± SEM, and circles represent data points for each animal (two-way ANOVA, $F_{Interaction}(1,25)= 0.1851$, $p = 0.6707$, Tukey's post hoc test).

10. Early-in-life bumetanide treatment rescues increased susceptibility to epileptic seizures in adult Ts65Dn mice

Deidda and colleagues reported no significant effect of bumetanide on the increased susceptibility to epileptic seizures, when the drug-treatment was performed in adult Ts65Dn mice. To assess whether treatment with bumetanide during a specific developmental window can rescue the higher susceptibility to seizures of trisomic mice in adulthood, we performed the audiogenic seizure (AGS) test in adult (P110-120) Ts65Dn and WT mice injected with vehicle or bumetanide from P2 to P15. In agreement with the literature (Westmark, Westmark et al. 2010, Deidda, Parrini et al. 2015), Ts65Dn vehicle-treated mice exhibited increased AGS susceptibility in comparison to WT, control mice, as assessed by the percentage of animals that exhibited AGS (Figure 41A), or mean AGS severity score (Figure 41B). Notably, bumetanide treatment during early postnatal life fully restored the increased AGS susceptibility in adult Ts65Dn mice.

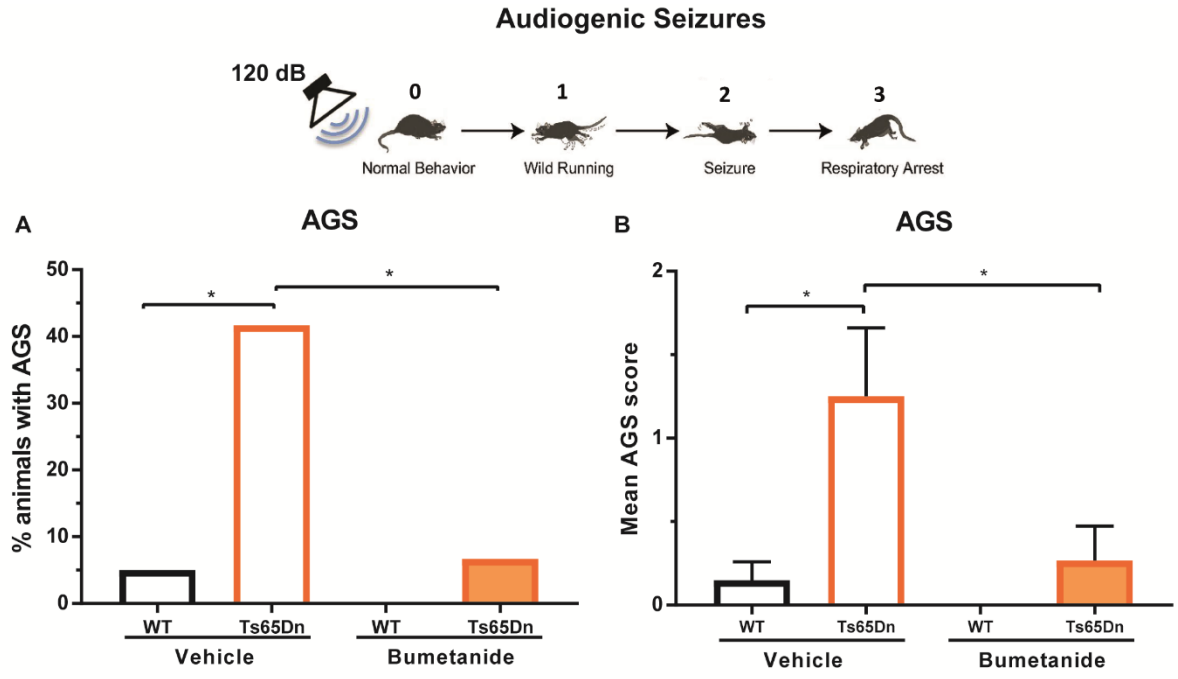


Figure 41. Early-in-life bumetanide treatment rescues increased susceptibility to seizures in adult Ts65Dn mice. Top, schematic representation of the AGS test and scoring. The numbers indicate the score given to an animal during the induction of a specific behavior by a 120 dB white noise. Bottom, **(A)** Percentage of animals that exhibited AGS susceptibility (left, Chi Square test with Sidak adjustment for multiple comparisons, **P<0.01, *P < 0.05), **(B)** mean AGS severity score, (two-way ANOVA on ranks, $F_{Treatment}$ (1,58)= 8.404, p = 0.0053, Student-Newman-Keuls Method, *P < 0.05). Bar charts represent the percentage of animals showing AGS (left) or average severity score ± SEM (right). The numbers of animals tested (WT-vehicle: 20, Ts65Dn-vehicle: 12, WT-bumetanide: 15, Ts65Dn-bumetanide: 15).

As for the novel object recognition and contextual fear conditioning tests, we found no gender-selective difference in the susceptibility to epileptic seizures of Ts65Dn mice (Figure 42). Female trisomic vehicle-treated animals showed significantly higher score in audiogenic seizures in comparison to WT females, while male trisomic animals showed a clear trend for increased AGS score. For both genders, bumetanide treatment improved AGS score in Ts65Dn mice. In WT animals no significant phenotype was found in both males and females treated with bumetanide.

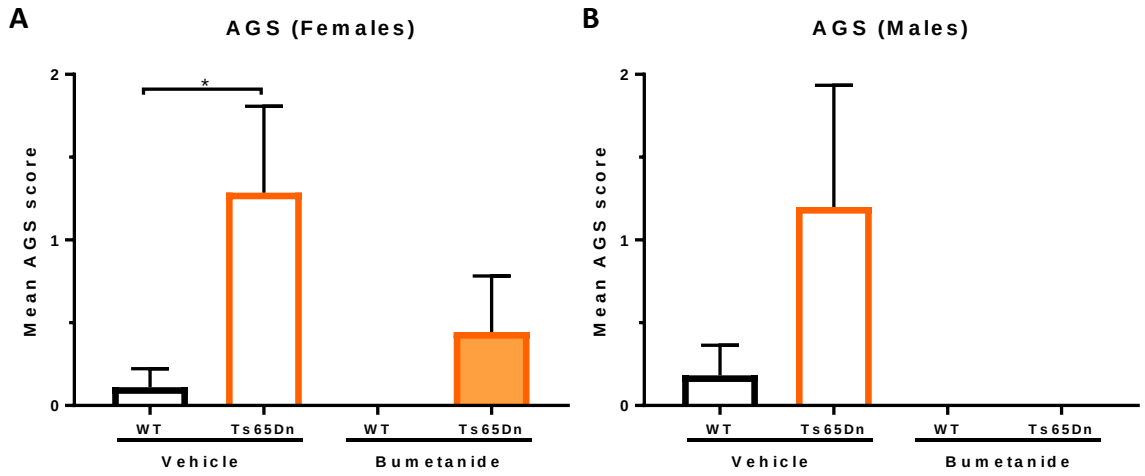
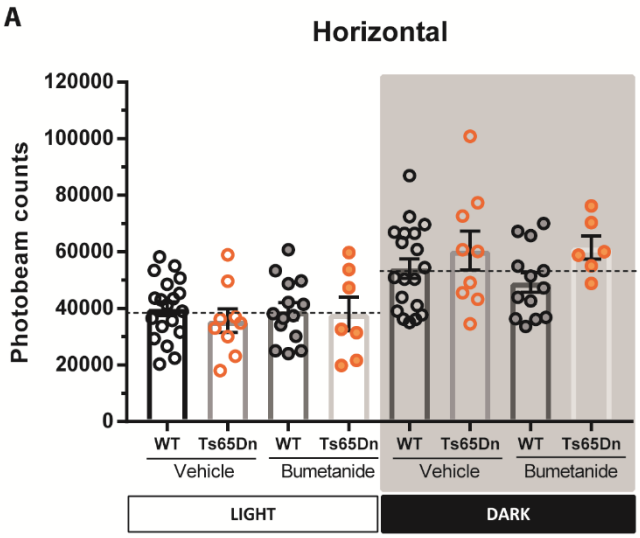


Figure 42. No sex-specific difference in the susceptibility to seizures in adult Ts65Dn mice. (A) Mean AGS severity score in female mice (two-way ANOVA on ranks, $F_{Genotype}$ (1,30)= 7.742, p = 0.0092, Student-Newman-Keuls Method). Bar charts represent the average severity score ± SEM (right). The numbers of female animals tested (WT-vehicle: 9, Ts65Dn-vehicle: 7, WT-bumetanide: 9, Ts65Dn-bumetanide: 9). **(B)** Mean AGS severity score in male mice (two-way ANOVA on ranks, $F_{Treatment}$ (1,24)= 4.617, p = 0.0420, Student-Newman-Keuls Method). Bar charts represent the average severity score ± SEM (right). The numbers of male animals tested (WT-vehicle: 11, Ts65Dn-vehicle: 5, WT-bumetanide: 6, Ts65Dn-bumetanide: 6).

11. Early-in-life intervention alters locomotor activity in mice

Deidda and colleagues reported no significant effect of bumetanide on the increased locomotor activity in Ts65Dn animals (Reeves, Irving et al. 1995) when bumetanide was given in adult trisomic mice (Deidda, Parrini et al. 2015). To assess whether early-in-life bumetanide treatment could rescue the increased locomotor activity in trisomic mice (Reeves, Irving et al. 1995), we monitored the locomotor activity of adult WT and Ts65Dn animals treated with vehicle and bumetanide as pups during the first two postnatal weeks of life over the course of 24 hours. For the analysis, the 24-hour monitoring was divided in 12 hours of light and 12 hours of dark. We found no significant differences between groups in the light phase for all the parameters analyzed (horizontal activity, total distance travelled and vertical activity), which is in agreement with the literature (Deidda, Parrini et al. 2015). During the dark phase, we found no significant difference among groups. Nevertheless, both Ts65Dn groups (treated with vehicle and bumetanide) showed a trend for increased locomotor activity during the dark phase for horizontal activity, total distance travelled and vertical activity (Figures 42A,B,C) in comparison to WT controls. Ts65Dn animals have a trend to significant activity compared to WT mice, but the number of animals is too low to draw final conclusions. No significant effect of bumetanide treatment was also observed in any group.



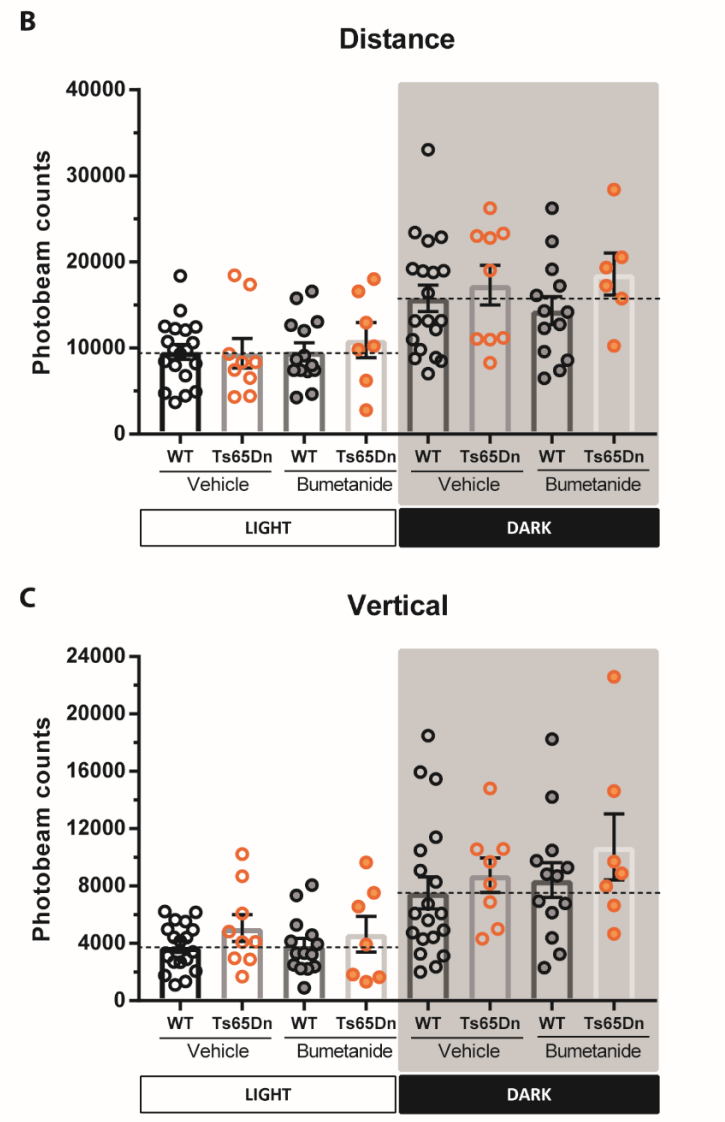


Figure 43. Early-in-life bumetanide treatment does not affect locomotor activity in Ts65Dn adult mice. (A) Quantification of the horizontal locomotor activity of mice treated with vehicle or bumetanide from P2 to P15. The light phase is represented on the left, whereas the dark phase is represented shadowed on the right. Bars represent the average locomotor activity ± SEM, and circles represent data points for each animal. Light phase: two-way ANOVA, $F_{Interaction}$ (1,45)= 0.1937, p = 0.6620, Tukey's post hoc test, * p < 0.05, two-way. Dark phase: two-way ANOVA, $F_{Interaction}$ (1,43)= 0.3904, p = 0.5354, Tukey's post hoc test, * p < 0.05, two-way. **(B)** Quantification of the total distance travelled in mice treated with vehicle or bumetanide from P2 to P15. The light phase is represented on the left, whereas the dark phase is represented shadowed on the right. Bars represent the average locomotor activity ± SEM, and circles represent data points for each animal. Light phase: two-way ANOVA, $F_{Interaction}$ (1,45)= 0.3264, p = 0.5707, Tukey's post hoc test, * p < 0.05, two-way. Dark phase: two-way ANOVA, $F_{Interaction}$ (1,43)= 0.4262, p = 0.5173, Tukey's post hoc test, * p < 0.05, two-way. **(C)** Quantification of the vertical locomotor activity of mice treated with vehicle or bumetanide from P2 to P15. The light phase is represented on the left, whereas the dark phase is represented shadowed on the right. Bars represent the average locomotor activity ± SEM, and circles represent data points for each animal. Light phase: two-way ANOVA, $F_{Interaction}$ (1,44)= 0.1239, p = 0.7266,

Tukey's post hoc test, * $p < 0.05$, two-way. Dark phase: two-way ANOVA, $F_{Interaction}(1,43)= 0.1353$, $p = 0.7148$, Tukey's post hoc test, * $p < 0.05$, two-way.

We analyzed males and females separately to assess any gender difference in the performance of the groups. No significant differences were found among groups (Figure 44).

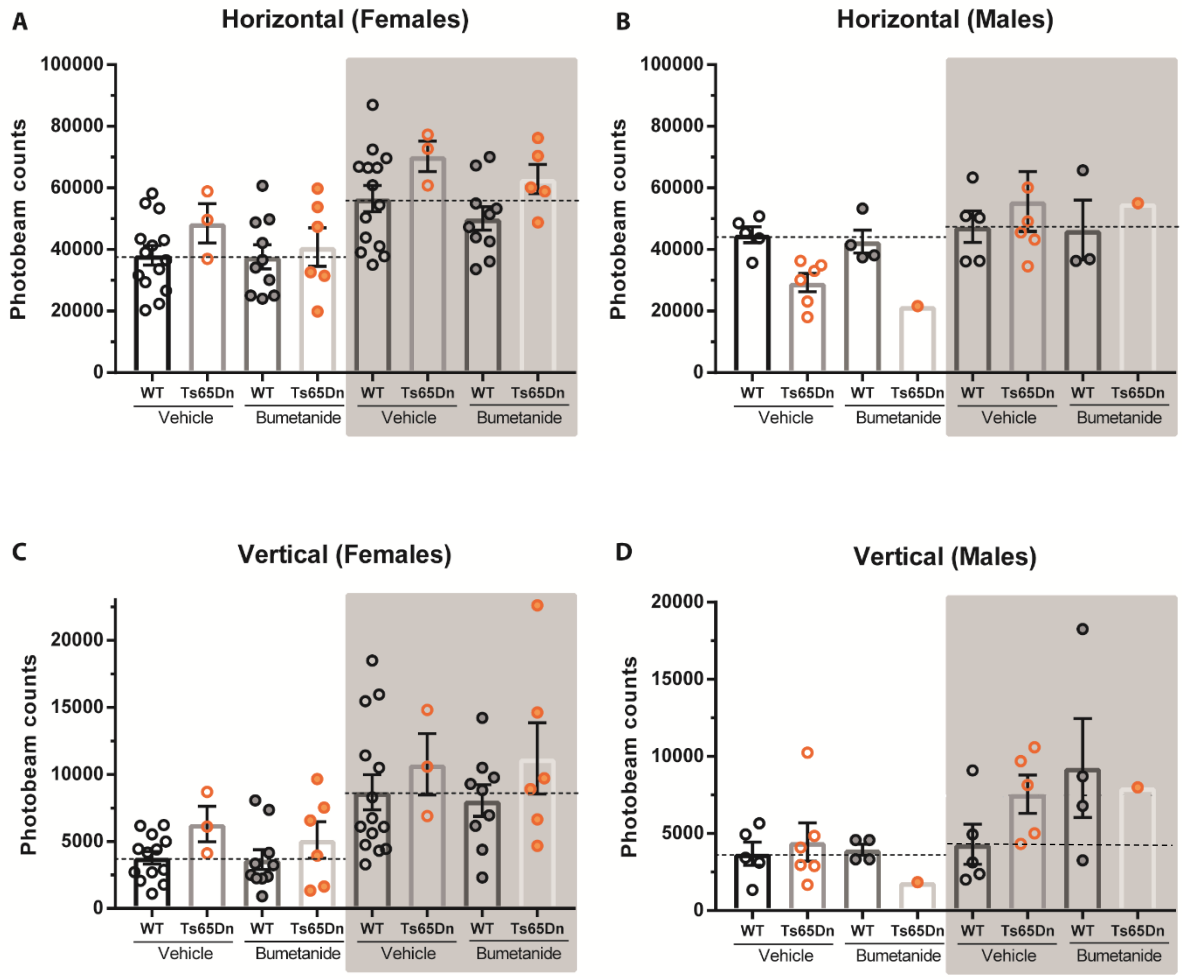


Figure 44. Early-in-life bumetanide treatment does not have a significant gender-specific locomotor activity in Ts65Dn adult mice. (A) Quantification of the horizontal locomotor in female mice treated with vehicle or bumetanide from P2 to P15. The light phase is represented on the left and whereas the dark phase is represented shadowed on the right. Bars represent the average locomotor activity ± SEM, and circles represent data points for each animal. Light phase: two-way ANOVA, $F_{Genotype}(1,29)= 1.712$, $p = 0.2010$, Tukey's post hoc test, * $p < 0.05$, two-way. Dark phase: two-way ANOVA, $F_{Genotype}(1,28)= 5.445$, $p = 0.0270$, Tukey's post hoc test, * $p < 0.05$, two-way. **(B)** Quantification of the horizontal locomotor in male mice treated with vehicle or bumetanide from P2 to P15. The light phase is represented on the left and whereas the dark phase is represented shadowed on the right. Bars represent the average locomotor activity ± SEM, and circles represent data points for each animal. Light phase: two-way ANOVA, $F_{Genotype}(1,12)= 17.84$, $p = 0.0012$, Tukey's post hoc test, * $p < 0.05$, two-way. Dark phase: two-way ANOVA, $F_{Genotype}$

(1,11)= 0.4777, p = 0.5038, Tukey's post hoc test, * p < 0.05, two-way. **(C)** Quantification of the vertical locomotor activity in female mice treated with vehicle or bumetanide from P2 to P15. The light phase is represented on the left and whereas the dark phase is represented shadowed on the right. Bars represent the average locomotor activity ± SEM, and circles represent data points for each animal. Light phase: two-way ANOVA, $F_{Genotype}$ (1,28)= 4.396, p = 0.0452, Tukey's post hoc test, * p < 0.05, two-way. Dark phase: two-way ANOVA, $F_{Genotype}$ (1,28)= 1.702, p = 0.2026, Tukey's post hoc test, * p < 0.05, two-way. **(D)** Quantification of the vertical locomotor activity in mice treated with vehicle or bumetanide from P2 to P15. The light phase is represented on the left and whereas the dark phase is represented shadowed on the right. Bars represent the average locomotor activity ± SEM, and circles represent data points for each animal. Light phase: two-way ANOVA, $F_{Genotype}$ (1,12)= 0.2331, p = 0.6380, Tukey's post hoc test, * p < 0.05, two-way. Dark phase: two-way ANOVA, $F_{Genotype}$ (1,11)= 0.1386, p = 0.7168, Tukey's post hoc test, * p < 0.05, two-way.

12. Early-in-life bumetanide treatment rescues $GABA_A$ergic signaling in hippocampal acute slices of adult Ts65Dn mice

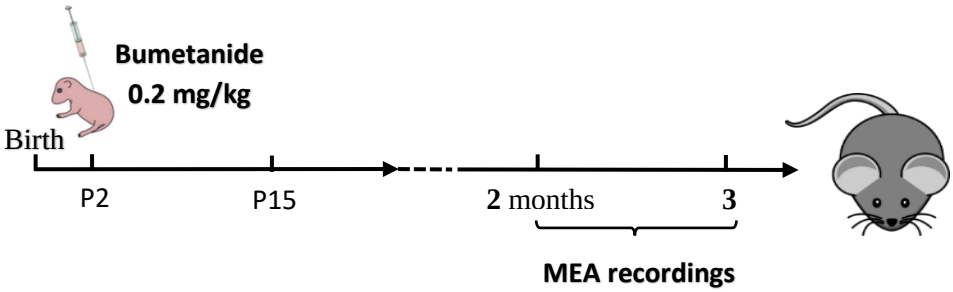


Figure 45. Schematic representation of the experimental protocol for WT and Ts65Dn mice. WT and Ts65Dn pups were treated with either vehicle or bumetanide for the period from P2 until P15. Mice were weaned at P28 and electrophysiological recordings were performed between P60-90.

We then investigated on the possible mechanisms underlying the behavioral rescue in long-term memory (NOR and CFC) and susceptibility to epileptic seizures upon bumetanide treatment early-in-life in Ts65Dn animals. We assessed whether the bumetanide treatment could rescue the increased GABA-mediated spiking events already described in adult Ts65Dn mice (Deidda, Parrini et al. 2015). To this aim, we treated WT and Ts65Dn pups with vehicle or bumetanide from P2 to P15, and performed multi-electrode array recordings (MEA) in acute brain slices (at >P70) from the previously treated animals. Hippocampal slices were recorded at basal conditions for 30 minutes followed by 30 minutes of recordings after exogenous GABA (100 μM) administration in the bath. As expected, the vehicle-treated WT mice showed a suppression of average MFR (with a values near to zero) (Figure 32A). Interestingly, slices from WT animals treated with bumetanide as pups did not significantly change their MFR ratio in comparison to WT vehicle-treated slices.

Conversely, vehicle-treated Ts65Dn (Figure 32A) showed significantly increased MFR ratio upon GABA administration in comparison to WT vehicle-treated animals. Remarkably, early-in-life bumetanide treatment significantly restored the response to exogenous GABA bath application in adult Ts65Dn hippocampal slices (Figure 32A).

Then, we quantified the percentage of electrodes for each slices showing significant change in comparison to their baseline level (threshold of 10% change). We found that the majority of the electrodes decreased their firing rate upon GABA application in vehicle-treated WT slices (16% of increasing electrodes, 69% of decreasing electrodes) or bumetanide-treated slices (30% of increasing electrodes, 50% of decreasing electrodes) (Figure 32B). Conversely, in vehicle-treated Ts65Dn slices the majority of the active electrodes increased the average MFR level upon exogenous GABA administration (67% of increasing electrodes, 17% of decreasing electrodes). However, Ts65Dn slices from mice treated with bumetanide from P2 until P15 displayed a significant decrease in the MFR with a percentage of MFR variation comparable to that of vehicle-treated WT (46% of increasing electrodes, 47% of decreasing electrodes), indicating a significant rescue of the inhibitory GABAergic signaling.

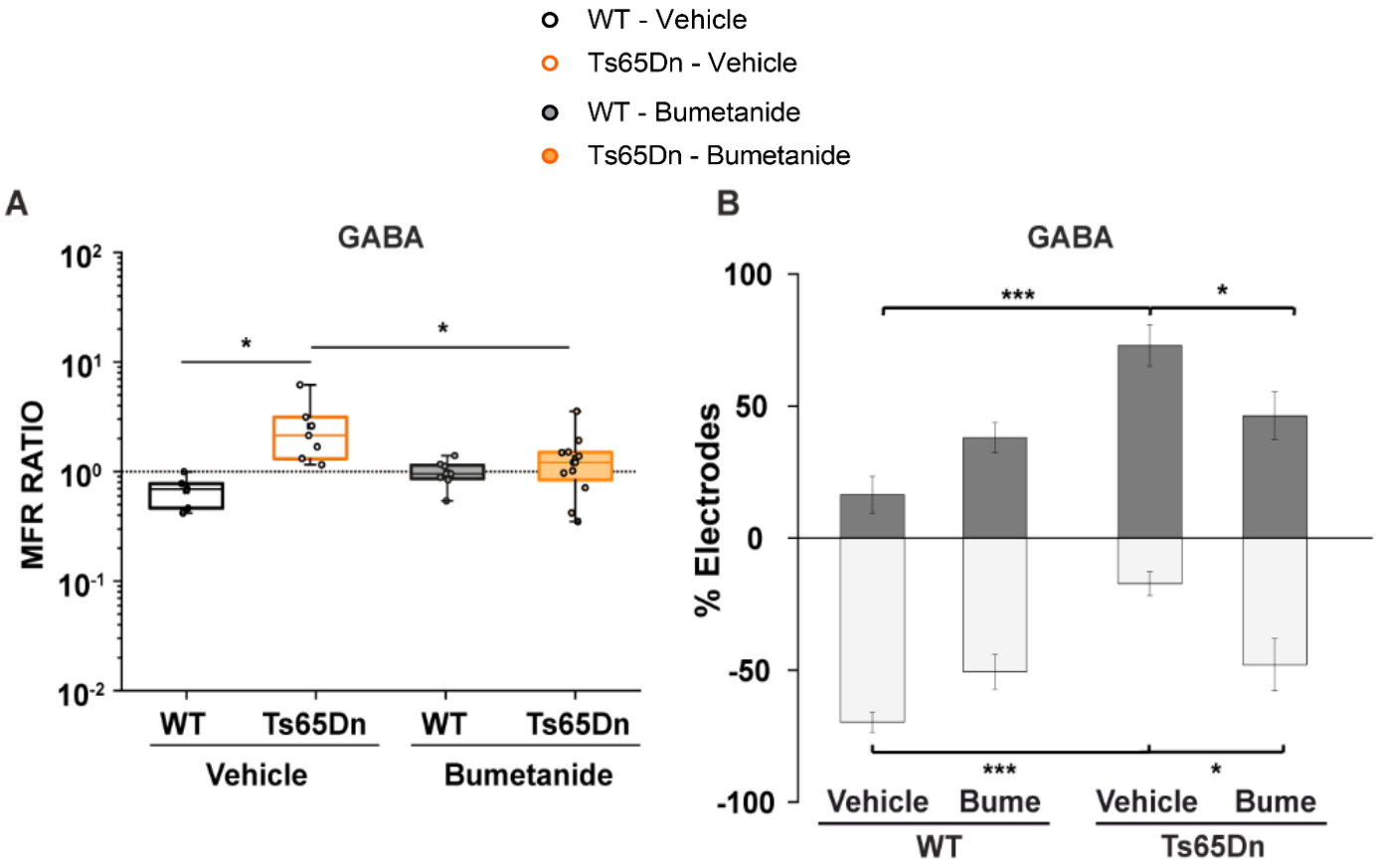


Figure 46. Early-in-life bumetanide treatment rescues GABAergic signaling in adult Ts65Dn mice. Hippocampal multi-electrode array recordings (MEA) from (8-12 weeks old) adult WT and Ts65Dn mice treated with vehicle or bumetanide from P2 until P15. **(A)** Quantification of MFR ratio after exogenous GABA administration in the bath. MFR for each electrode is normalized to the value of the same electrode during basal condition (indicated as the dotted line) and then average for each slice. The small square indicates the mean, the central line illustrates the median, and the box limits indicate the 25th and 75th percentiles and each dot represents the MFR ratio for each recorded brain slice. Bars represent the MFR of all recorded electrodes ± SEM, and dots represent data points for each slice (WT-vehicle: 2 animals, 6 slices Ts65Dn-vehicle: 3 animals, 8 slices, WT-bumetanide: 3 animals, 8 slices, Ts65Dn-bumetanide: 5 animals, 12 slices). Data points falling on the dashed line (MFR equal to 0) show no change in the MFR, data points falling above the dashed line (MFR ratio >1) show an increase in MFR, and data falling below the dashed line (MFR ratio <1) show a decrease in MFR after drug administration in the bath (two-way ANOVA, *p<0.05; followed by Tukey's post hoc test). **(B)** Quantification of the percentage of MEA electrodes for each slice that significantly change the MFR after bath application of GABA. Bars above zero (dark grey) represent percentage of electrodes that increased their activity in comparison to the baseline after GABA application in the bath, whereas bars below zero (light grey) represent percentage of electrodes that decreased their activity in comparison to the baseline (two-way ANOVA, *p<0.05; **p<0.01; *** p<0.001; followed by Tukey's post hoc test).

Discussion

In this study, we demonstrate for the first time that an increased ratio of NKCC1/KCC2 is present since early postnatal life in trisomic mice and that interference with NKCC1 during a vulnerable developmental window of brain maturation resulted in lasting rescue of cognitive deficits, hyperactivity and susceptibility to seizure phenotypes in the adult Ts65Dn mice. To target NKCC1 we used a neuron-specific genetic approach by AAV9-mediated NKCC1 knockdown and systemic subcutaneous administration of the FDA-approved diuretic bumetanide, an NKCC1 inhibitor, during the first 2 weeks of development.

Chloride transporter expression is altered since early postnatal life in Ts65Dn mice

Here, we studied the developmental expression of the two cotransporters during brain development in WT animals and Ts65Dn mice. We found that both NKCC1 and KCC2 increase their expression in the hippocampus after birth in both WT and trisomic mice, as assessed at P2, P7, P15, P21, P30, >P60. . The robust upregulation of KCC2 is many-fold higher the upregulation of NKCC1. This is in line with evidence from the literature supporting the upregulation of KCC2 in many studies in both rodents (Rivera, Voipio et al. 1999, Kaila, Price et al. 2014, Watanabe and Fukuda 2015) and humans (Vanhatalo, Palva et al. 2005, Sedmak, Jovanov-Milosevic et al. 2016). This also points to the robust upregulation of KCC2 postnatally as the main mediator of the GABA polarity switch (Rivera, Voipio et al. 1999). This is in line with the fact that premature overexpression of KCC2 early in development results in a physiological (and not hyperpolarized) ECl^- (Cancedda, Fiumelli et al. 2007). In contrast, RNA interference for NKCC1 knock down *in utero* reversed E_{GABA} at more hyperpolarizing potentials in neocortical neurons than in controls (Wang and Kriegstein 2008). On the other hand, the developmental regulation of NKCC1 mRNA and/or protein has been a topic of controversy with a series of human and rodent (both rats and mice) studies reporting either a clear developmental upregulation (Yan, Dempsey et al. 2001, Wang, Shimizu-Okabe et al. 2002, Hyde, Lipska et al. 2011, Sedmak, Jovanov-Milosevic et al. 2016), or developmental downregulation (Shimizu-Okabe, Yokokura et al. 2002, Yamada, Okabe et al. 2004, Dzhala, Talos et al. 2005) or stable expression (Mao, Garzon-Muvdi et al. 2012) during development possibly. This was possibly due to technical difficulties identifying NKCC1 that can

be mediated by epitope masking after epitope phosphorylation (Delpire and Austin 2010, Hartmann and Nothwang 2014) and/or unintended splice isoform specificity of NKCC1 available antibodies (Puskarjov, Kahle et al. 2014, Virtanen, Uvarov et al. 2020). Moreover, sequence diversity of NKCC1 homologues across species (98% between mice and rats and 94% between mice and humans) should be taken into account considering that the differences between species are mainly located in N-terminal parts and C-terminal regions around exon 21, a region which is alternatively spliced into NKCC1a and NKCC1b isoforms (Randall, Thorne et al. 1997, Hebert, Mount et al. 2004, Virtanen, Uvarov et al. 2020). On the other hand, since NKCC1, unlike KCC2, is expressed not only in neurons, it is possible that the discrepancy in the diverse studies is given by a diverse contribution from astrocytes and oligodendrocytes, which express NKCC1 continue to proliferate in the postnatal brain (Annunziato, Boscia et al. 2013, Reemst, Noctor et al. 2016).

In particular, we found that Ts65Dn mice have significantly upregulated NKCC1 protein at P7, P21, >P60 in comparison to WT mice , while we found a significant downregulation of KCC2 at P2, P15, P30 in trisomic hippocampi in comparison to WT animals. Computing the ratio of NKCC1 to KCC2 we found that Ts65Dn have an altered Cl^- transporter ratio at all the time points studied, with P2 being the first time point studied. We expressed the NKCC1/KCC2 ratio as a reference for prediction of E_{GABA} in neurons. The assumption is that since the two transporters play opposite roles in Cl^- transportation, a decreasing NKCC1/KCC2 ratio during development should be in line with the described excitatory to inhibitory shift in GABAergic signaling sustained by the increasing levels of negative shift in E_{GABA} values. Nevertheless, we are currently validating this assumption by electrophysiological recordings also in light of the already mentioned expression of NKCC1 by astrocytes and oligodendrocytes.

Our findings on increased NKCC1/KCC2 ratio during development in Ts65Dn mice are supported by recent data showing a delay in GABA polarity switch in primary hippocampal cultures and acute hippocampus slices of Ts65Dn mice. There, authors found that the timing of the GABA switch was delayed by 2–3 days in Ts65Dn hippocampal cultures and by 2 days in CA3 slices. However, they did not investigate the molecular mechanisms undelaying this delay (Lysenko, Kim et al. 2018). Our results on the developmental expression of NKCC1 and KCC2 showing significantly increased NKCC1/KCC2 ratio at all the time points studied come from bulk

hippocampal samples. This doesn't allow us to draw conclusions about possible sub-regional differences in cotransporter expression.

Finally, considering that the upregulation of NKCC1 described in adult Ts65Dn mice by Deidda and colleagues was also confirmed in the hippocampus of adult DS people (Deidda, Parrini et al. 2015) and that our results show a significantly increased ratio of NKCC1 to KCC2 present early on in postnatal Ts65Dn hippocampus, future investigations should study whether alterations in cotransporter regulation is present during brain development also in DS people.

Early interventions targeting NKCC1 result in long-term rescue of deficits in adult Ts65Dn mice

Our results suggest that early interventions targeting NKCC1 have long-term effects in rescuing learning and memory, hyperactivity, susceptibility to epileptic seizures and GABAergic signaling in the adult Ts65Dn mice. A long-lasting effects of a transient treatment with bumetanide during development has been also reported by other groups in epilepsy (Marguet, Le-Schulte et al. 2015), Fragile X (Tyzio, Nardou et al. 2014, He, Arroyo et al. 2019) and Rett syndrome (Lozovaya, Nardou et al. 2019). In particular, a study using Kv7 current-deficient mice, a model of neonatal epileptic encephalopathy, has shown that bumetanide treatment between P0-P14 prevents structural and behavioral pathology and normalizes network activity in these mice (Marguet, Le-Schulte et al. 2015). Moreover, in Fragile X mice (Fmr1 KO mice) the authors reported defective E_{GABA} in the hippocampus due to downregulation of KCC2. Notably, maternal pretreatment with bumetanide rescued electrophysiological and behavioral phenotypes in offspring though an oxytocin signaling-dependent mechanism (Tyzio, Nardou et al. 2014). More recently, He and colleagues suggested an alternative model for the altered E_{GABA} in Fragile X mice, showing elevated NKCC1 expression in the postnatal cortex of Fmr1 KO mice resulting in synaptic deficits and circuit dysfunction that were rescued by bumetanide treatment (P0-P10) with lasting effects (He, Arroyo et al. 2019). Tyzio and colleagues did not investigate NKCC1 levels in Fmr1 KO hippocampus and He and colleagues did not find differences in KCC2 expression levels in mutants. The different etiology from chloride dysregulation in the two studies in Fmr1 KO mice could be explained by region specific differences in cotransporter expression. However, both models point

to an increased NKCC1/KCC2 ratio and the positive outcome of bumetanide treatment. Moreover, in CA3 pyramidal neurons of Rett syndrome mice (MeCP2 mutant) the developmental GABAergic polarity shift was reported to be abolished and responses to GABA were excitatory two weeks after birth, which was restored by maternal bumetanide treatment one day before delivery (Lozovaya, Nardou et al. 2019). Common ground between these studies and ours is the lasting effect of early bumetanide treatment in rescuing disrupted developmental processes.

Considering the rescue of long-term memory (novel object recognition, contextual fear conditioning and object location), the effect that we found in animals infected with the neuron-specific AAV9-NKCC1 amiR at P2 could be simply the result of NKCC1 downregulation during the period of testing (adulthood). This is because NKCC1 knock down by AAV9-NKCC1 amiR likely lasts throughout adulthood (Ittner, Klugmann et al. 2019) and after adult bumetanide treatment is sufficient to rescue cognitive impairment in Ts65Dn animals (Deidda, Parrini et al. 2015). On the other hand, our approach by early-in-life treatment with bumetanide (P2-P15) indicated that a transient interference with NKCC1 function during brain maturation can *per se* have long-lasting effects on Ts65Dn adult mice rescuing long-term memory in the novel object recognition task and contextual fear conditioning. Nevertheless, when we tested the same animals in the object location task, we found that vehicle-treated WT animals showed a poor discrimination index. A gender-specific analysis revealed that the controls animals that performed poorly in the object location test were males. This is in line with data from the literature showing that early life stress (ELS) has long-lasting, gender-selective effect on the performance of male mice specifically in the object location task during adulthood (Naninck, Hoeijmakers et al. 2015, Bath, Nitenson et al. 2017). Indeed, because of the poor pharmacokinetics of bumetanide we followed a two injection a day strategy as previously described (Deidda, Allegra et al. 2015, Marguet, Le-Schulte et al. 2015). This was possibly perceived as an ELS by pups. Interestingly, the same study showed that the negative effect of ELS in cognitive performance of female mice was also observed in young animals (P21 and P38), but did not persist in early adulthood (Bath, Nitenson et al. 2017). This is again in line with the good discrimination index that we found in the task in female mice, as tested between P90-P120. Of note, the gender-selective impact of ELS in spatial memory has been described also in the Morris water maze task (Naninck, Hoeijmakers et al. 2015). Although we did not test our experimental animals in the Morris water maze, we did found poor spatial memory

performance in male controls in the object location test supporting the gender-selective impairment of ELS in spatial memory.

Interestingly, early-in-life NKCC1 knock down rescued short-term memory in the T-maze task in adult Ts65Dn mice, which is in line with data showing the same rescue after selective NKCC1 inhibition in young adult trisomic (male) animals (Savardi, Borgogno et al. 2020). However, gender-depended analysis of animals treated with vehicle as pups revealed a male-selective impairment of adult Ts65Dn mice in short-term memory (T-maze task). Conversely, Faizi et al. did show impaired spontaneous alteration of female Ts65Dn in the T-maze task. These seemingly contradicting results may depend on subtle differences in the protocol. Indeed, Faizi et al themselves reported no deficit in spontaneous alteration in both male and female Ts65Dn mice in Y-maze, a task very similar to the T-maze and also assessing short-term memory (Faizi, Bader et al. 2011). Notably, in the T-maze task, the discrepancy between the rescue of short-term memory deficit seen in Ts65Dn mice after early-in-life NKCC1 knock down and the no effect after early-in-life bumetanide treatment could be explained by the lifelong downregulation of NKCC1 with the early-in-life AAV9 NKCC1 interference approach, suggesting that transient NKCC1 inhibition by bumetanide treatment might not be enough for the rescue of this deficit in the adult Ts65Dn animals.

Both early downregulation of NKCC1 starting at P2 and early-in-life bumetanide treatment resulted in a complete rescue of the susceptibility to seizure phenotype, almost three months after the treatment cessation in Ts65Dn mice. Adult bumetanide treatment did not rescue the susceptibility to seizures in trisomic mice (Deidda, Parrini et al. 2015). This suggests that additional mechanisms independent of depolarizing GABA signaling or early circuit alterations underlie the epileptic phenotype in DS animals. Interestingly, in a genetic rat model of epilepsy (GERP) increased susceptibility to audiogenic seizures has been attributed to an increased number of GABAergic neurons (as determined by GAD immunoreactivity) in circuits of the inferior colliculus (IC) (Faingold, Gehlbach et al. 1986, Maxson 2017, Ribak 2017). However, the IC neurons in GERP rats were found to be less sensitive to GABA and benzodiazepine iontophoresis than in control rats (Faingold, Gehlbach et al. 1986). Moreover, a rat study showed that susceptibility to audiogenic seizures resulted from neuronal hyperexcitability to sound induced by a decreased GABAergic inhibition in IC (Bagri, Sandner et al. 1989). Although the authors claimed

a decreased effectiveness of inhibition in the IC of audiogenic susceptible rats (Faingold 2002), no recordings of E_{GABA} were performed in these studies. The increased susceptibility seen in Ts65Dn mice (Westmark, Westmark et al. 2010, Deidda, Parrini et al. 2015) might be related to altered GABAergic signaling in these same local circuits. Transient interference with bumetanide early-in-life could rescue the pathological trajectories in adulthood. Additionally, the susceptibility to seizures in adult Ts65Dn animals was also rescued by neuron-specific AAV9-mediated NKCC1 knockdown early-in-life. Notably, injection of the AAV9 construct at P2 resulted in a large transduction brain-areas in adulthood including hippocampus, cortex, and subcortical regions that could also explain the rescue of increased seizure phenotype and the networks mediate this phenotype. These data support that timed interference with NKCC1 during development is vital for the prevention of susceptibility to seizure phenotype in adult trisomic animals.

The hyperactivity phenotype was rescued by early downregulation of NKCC1. Also this deficit was not ameliorated by bumetanide treatment in adult DS animals (Deidda, Parrini et al. 2015), suggesting again that it might arise during development and be encoded in the circuits. Interestingly, hyperactivity is highly comorbid in neurodevelopmental disorders (Gnanavel, Sharma et al. 2019). Although fMRI studies have revealed both shared and distinct functional networks between ADS and ADHD subjects (Di Martino, Zuo et al. 2013) more studies are needed to better understand comorbidity in NDs and circuit specific behavioral deficits. Notably, we could not see the known increased locomotor activity of vehicle-treated Ts65Dn in comparison to WT animals, when animals were injected twice a day postnatally. This was due to the fact that vehicle-treated WT mice as pups showed themselves increased locomotor activity in the dark phase in comparison to control amiR animals in this study and as described by Deidda and colleagues. Possibly, ELS could have a role also in altered locomotor activity. However, the low number of animals that tested does not allow to draw us final conclusions about gender-selective ELS impact due to early-in-life injections, yet. Overall, the controversy over the impact of ELS on cognition depends widely on the stress induction protocol (Buckert, Schwieren et al. 2014). Moreover, each learning and memory task involves different brain areas and the effects of ELS could be region or circuit specific depending on the kind of stressor and time of the induction. We are currently running experiments using pharmacological intervention by oral administration of bumetanide in pups (by treating their dam with bumetanide in the water) to try to avoid the adverse selective ELS impact by repeated pup early-in-life injections.

Interestingly, our results show that WT animals injected with NKCC1 amiR or treated with bumetanide early-in-life do not show any significant phenotype in any behavioral test. This is in line with He and colleagues that found no alterations of brain and cognitive development in control littermates treated with bumetanide the same period as we did (He, Arroyo et al. 2019). The fact that we did not see any phenotype after early NKCC1 interference in WT animals, despite the fundamental role that depolarizing GABAergic signaling plays during development, could be explained by strong compensation for the lack of Cl^- uptake in the network activity described in the neonatal hippocampus of NKCC1 $^{-/-}$ animals (Sipila, Huttu et al. 2009). In line with our results these compensatory mechanisms are in place also under more subtle conditions of NKCC1 downregulation as seen *in vivo* with conditional loss of NKCC1. In particular, in mice with conditional loss of NKCC1 in telencephalic glutamatergic neurons, although impacted hippocampal activity in slices by impairing neuronal synchrony, *in vivo* had a minor impact on spontaneous hippocampal activity with no significant deficits found in hippocampus-dependent behaviors (Graf, Zhang et al. 2020). It is possible that in WT animals strong compensatory mechanisms after interference with NKCC1 during development result in no behavioral abnormality in adulthood. It is also possible that dysregulation of NKCC1/KCC2 in the context of DS, where trisomy leads to dysregulation in cellular and molecular levels in multiple systems the capacity for compensation is reduced and behavioral alterations are manifested.

It is evident from our results that early-in-life interference with NKCC1 results in lasting effects in adulthood. We are currently investigating the mechanisms underlying this long-lasting effect. Interestingly, a previous study (Deidda, Allegra et al. 2015) demonstrated that early interference of NKCC1 with bumetanide treatment (between P3-P8) in rats extended the duration of the critical period of the visual cortex plasticity into young adulthood (P35). BDNF is known to regulate the closure of the critical period in the visual system and studying changes in plasticity markers and reduced inhibitory tone revealed the effect of bumetanide on critical period prolongation. Notably, the authors found reduced density of perineuronal nets, down-regulation of BDNF and decreased frequency of mIPSCs at P35 that did not persist though in adulthood. Moreover, early bumetanide treatment did not alter the overall structural development of the visual system (Deidda, Allegra et al. 2015). In the case of DS, conflicting results on BDNF expression come from a number a studies with some reporting no difference in expression levels between trisomic and WT animals (Lockrow, Boger et al. 2011, Kleschevnikov, Belichenko et al. 2012, Begenisic, Sansevero et al.

2015, Parrini, Ghezzi et al. 2017), while others reporting reduced hippocampal expression in Ts65Dn mice at different ages (Bianchi, Ciani et al. 2010, Voss, Heo et al. 2013, Stagni, Giacomini et al. 2015). The reported discrepancies possibly come from age, gender and structure differences. In the Ts65Dn mice overproduction of parvalbumin interneurons has been observed in the hippocampus (Chakrabarti, Best et al. 2010), however a human study found reduced parvalbumin-positive interneurons in the frontal and temporal cortices of DS patients (Kobayashi, Emson et al. 1990). Future studies should investigate the role of GABAergic interneurons in DS. Finally, less is known about the perineuronal nets of the extracellular matrix in the Ts65Dn mice with one study reporting increased deposition of versican V2 in the stratum oriens of the hippocampus (Howell and Gottschall 2012). Considering these studies and the effect that Deidda and colleagues reported after bumetanide treatment further investigation should better characterize plasticity markers in DS and the effect of early interference with NKCC1. In Fragile X mice, bumetanide treatment during early postnatal life (P0-P10) resulted in lasting rescue of LTP and whisker response maps in barrel cortex in the adult Fmr1 KO mice. The authors attributed the long-term effects of early bumetanide treatment to the proteome remodeling they found in mice treated. Notably, they found increased levels of levels of parvalbumin (PV) and TrkB and restoration of MeCP2 and GAP43 expression levels after bumetanide treatment (He, Arroyo et al. 2019). Interestingly, Deidda and colleagues did not find alterations in parvalbumin-positive interneurons on the visual cortex of rat animals treated with bumetanide as pups. This could be explained due to different techniques used for detection, as He et al., reported protein PV upregulation using mass-spectroscopy and Deidda et al., investigated PV-immunoreactivity levels. Additionally, differences between bumetanide effects on mice and rats cannot be excluded.

Surprisingly, early pharmacological treatment with bumetanide during the first two postnatal weeks of life restored increased GABA-mediated spiking events upon GABA bath application in hippocampal slices of young adult trisomic animals (2-3 months old). This lasting effect of early-in-life bumetanide treatment in adulthood, three months after the treatment cessation clearly suggests the presence of an opportunity window for the correction of GABAergic signaling observed in adult trisomic animals (Deidda, Parrini et al. 2015). Early neuron-specific AAV9-mediated NKCC1 knockdown also rescued GABAergic signaling after both GABA and bicuculline administration in the bath, confirming that the increased NKCC1 mediates the increased GABA-mediated spiking activity. Furthermore, we observed that some slices of NKCC1

amiR WT group increased their MFR upon GABA application, but did not reach a significant difference with respect to WT control amiR, while the MFR upon bicuculline resulted in a significant decrease of MFR in slices of NKCC1 amiR WT group in comparison to NKCC1 amiR WT slices. A slight, but not significant increase of MFR upon GABA bath application was also observed in hippocampal slices of WT animals treated with bumetanide as pups. These activity alterations in GABAergic activity after early interference with NKCC1 in WT does not correlate with any significant behavioral phenotype in the behavioral battery performed. This could be due to biological robustness, subtle alterations in the network that do not lead to behavioral anomalies due to strong compensation observed also in the NKCC1 $^{-/-}$ mice (Sipila, Huttu et al. 2009, Graf, Zhang et al. 2020).

The need of early interventions to correct neurodevelopment in DS

A growing body of evidence indicates that neurodevelopmental disorders arise from alterations of the physiological developmental trajectories (Marin 2016, Del Pino, Rico et al. 2018, Chorna, Cioni et al. 2020). The necessity for early-in-life interventions in neurodevelopmental disorders is suggested both for syndromic and non-syndromic conditions. Those are characterized by early onset and developmental delays in reaching functional milestones regardless of the underlying cause of the ND (Meredith 2015).

In the case of Down syndrome, alterations in the brain are seen early during development, with decreased volume and reduced neuronal density in cortex, hippocampus and cerebellum (Contestabile, Fila et al. 2007, Patkee, Baburamani et al. 2020, Uguagliati, Al-Absi et al. 2021), defects of synaptic plasticity (Siarey, Stoll et al. 1997, Kleschevnikov, Belichenko et al. 2004, Costa and Grybko 2005) impaired hippocampus-dependent memory functions (Reeves, Irving et al. 1995, Contestabile, Greco et al. 2013) hyperactivity (Escorihuela, Fernandez-Teruel et al. 1995, Reeves, Irving et al. 1995, Sago, Carlson et al. 2000) increased susceptibility to seizures (Westmark, Westmark et al. 2010, Deidda, Parrini et al. 2015) and sleep disorders (Stewart, Persinger et al. 2007, Colas, Valletta et al. 2008, Das, Medina et al. 2015). In the clinical symptomatology, the cognitive impairment is the most common and severe feature of DS starting the first years of life. After the age of 40 the majority of DS individuals have histopathological

features of Alzheimer's disease , which deteriorates further the cognitive performance (Bull 2020). DS people do not only show developmental delays but also developmental regression, the loss of an acquired function or the failure to progress to the next developmental milestone beyond a prolonged plateau after a period of relatively normal development. In particular, the intelligence quotient (I.Q.) of Down syndrome individuals, which ranges from 40 (severe intellectual disability) to 70 (moderate) plateaus early-in-life and further declining in adolescence (Rachidi and Lopes 2008, Dierssen 2012). In this context, the possibility of prolongation of critical period plasticity by bumetanide treatment would possibly have a positive outcome in a case where cognition is declining. However, the study showing prolongation of CP after early bumetanide treatment was performed in WT rats (Deidda, Allegra et al. 2015) and further investigation in Ts65Dn mice should be performed. Moreover, the increased frequency of epileptic seizures has onset concentrated mostly early-in-life and aging, whereas hyperactivity starts in childhood even before the third year of life and lasts also in adult life (Green, Dennis et al. 1989) are often comorbid in DS people (Reeves, Irving et al. 1995, Westmark, Westmark et al. 2010, Ekstein, Glick et al. 2011, Dierssen 2012, Deidda, Parrini et al. 2015, Moss 2017). Our results demonstrating rescue of susceptibility to seizures and hyperactivity following early interventions targeting NKCC1 in Ts65Dn support early life interventions in DS to rescue epilepsy and hyperactivity deficits that are present since early in development. The developmental regression seen in DS subjects highlights even further that early-in-life (prophylactic in the case of DS) interventions should start as early as possible (Thurm, Powell et al. 2018). Indeed, considering that in humans, brain development is a process spanning more than two decades (from embryonic patterning to synaptic pruning and myelination; (Marin 2016, Silbereis, Pochareddy et al. 2016), this progressive decline in cognitive abilities could derive by either a time-specific perturbation at a given step during development or a secondary deficits arising from homeostatic, compensatory mechanisms acting over a protracted period due to earlier initial brain alterations.

Potential pitfalls of early therapeutic interventions

Bumetanide, is an FDA-approved diuretic, considered to be a very safe drug in adult. Most frequent side effects are due to its unselective action on NKCCs. Indeed, the diuretic effect of bumetanide depends on its ability to inhibit NKCC2 in the kidney, thus causing strong diuresis. Excessive

diuresis leads to secondary side effects that include, ionic imbalance. Nevertheless, ionic imbalance can be compensated with dietary supplements. In the developing brain, in fact bumetanide can induce ototoxicity by interfering with NKCC1 (expressed in the cochlea) (Delpire, Lu et al. 1999) and NKCC2 (in the endolymphatic sac at the membranous labyrinth) (Kakigi, Nishimura et al. 2009) signaling in developing the inner ear. Indeed, the NEMO trial (NCT01434225) which was investigating the use of bumetanide in newborns with hypoxic ischemic encephalopathy (HIE), was interrupted shortly after 3 out of 11 treated newborns were diagnosed with hearing loss (Pressler, Boylan et al. 2015). However, a recent clinical trial (NCT00830531) with bigger cohort and control group investigating the efficacy and safety of bumetanide treatment in newborns with HIE reported lower percentage of hearing impairment 8% of treated subjects (2 out of 26 subjects) *versus* 27% of treated subjects (3 out of 11 subjects) reported in the NEMO trial (Pressler, Boylan et al. 2015, Soul, Bergin et al. 2021). Notably, 4 out of 5 bumetanide-treated subjects that showed hearing impairment in both studies had HIE and received aminoglycoside (gentamicin) treatment, whose ototoxic effect has been reported to be aggravated by bumetanide (Zimmerman and Lahav 2013, Ben-Ari, Damier et al. 2016, Soul, Bergin et al. 2021). Therefore, the lower percentage of hearing impairment reported by Soul et al., could be partly attributed to the lower use of gentamicin in their trial (50% of HIE subjects treated *versus* 86% in the NEMO trial) (Soul, Bergin et al. 2021). Our results show no significant effect of early postnatal bumetanide treatment on the acoustic startle reactivity test, indicating no hearing loss in bumetanide-treated animals. The hearing loss after bumetanide treatment in newborns has been attributed to the synergetic effects of several factors such as immaturity of hair cells and differences between the roles of NKCC1 and KCC2 in the regulation of chloride in immature and adult cochlear hair cells (Brummett 1981, Milenkovic and Rubsamen 2011, Ben-Ari, Damier et al. 2016). In mice, responses to sounds can first be behaviorally observed and recorded from the auditory nerve around postnatal days 10 to 12, when the ear canal opens (Kros, Ruppersberg et al. 1998). However, the proliferation of hair cells occurs in embryonic life (E12-16) in mice (Marrs and Spirou 2012). Moreover, auditory nerve fibers generate spontaneous activity by E14 and drive third order central neurons by E17, nearly 2 weeks before hearing onset. Thus, functional establishment of the mouse auditory circuits occurs during late embryonic dates (Marrs and Spirou 2012), before the onset of our treatment protocol. This suggests that the lack on ototoxicity upon early postnatal treatment in mice could only reflect the differences between auditory system

development of humans and mice at birth. Further investigation of auditory system maturation and NKCC1 regulation in human development are therefore mandatory to shed light on the timing an intervention with bumetanide should follow not to jeopardize hearing ability. For example, in humans oral treatment bumetanide have been already given to children of an age ranging from 3 months to 18 years old to rescue behavioral deficits in autism (Lemonnier and Ben-Ari 2010, Lemonnier, Degrez et al. 2012, Lemonnier, Villeneuve et al. 2017). Currently, bumetanide treatment is being assessed for the safety and efficacy in two Phase III clinical trials in autistic children (2-7 years old) and adolescents (7-18 years old) (NCT03715153 and NCT03715166 respectively).

As already mentioned above, other side effects that have been investigated in mice include impairment in sensorimotor gating (Wang and Kriegstein 2011). However, these deleterious effects of embryonic inhibition of NKCC1 were not observed when bumetanide treatment began in the postnatal life (Wang and Kriegstein 2011). In addition, no adverse effect in terms of bumetanide treatment during the first two postnatal weeks have been reported by two studies on genetic epilepsy mouse model (Marguet, Le-Schulte et al. 2015) and maternal separation rat model (Hu, Yu et al. 2017). Early bumetanide treatment for a shorter period (P3-P8) in rats also did not have any acute adverse effect on body weight of treated pups (Deidda, Allegra et al. 2015). In line with the safety of postnatal treatment with bumetanide in mice, we found no adverse effect of early bumetanide treatment (P2-P15) in the body weight, of both WT and Ts65Dn treated mice. In humans, no adverse effects (besides strong diuresis and ionic imbalance) by early bumetanide treatment was also reported in a clinical trial with five autistic infants treated with bumetanide for three months (Lemonnier and Ben-Ari 2010) and chronic bumetanide treatment in autistic children and adolescents (2-18 years old) (Lemonnier, Degrez et al. 2012, Lemonnier, Villeneuve et al. 2017).

Finally, new, more potent and selective drugs may provide an alternative to bumetanide by reducing the side effects of the diuretic action of bumetanide. In particular, a novel selective NKCC1 inhibitor (no inhibition on NKCC2) has been developed and tested for its ability to rescue cognitive impairment in Ts65Dn mice, without a diuretic effect are already a promising therapeutic approach (Savardi, Borgogno et al. 2020). Moreover, a combined therapy, regulating the function of NKCC1 and KCC2 with the use of bumetanide and KCC2 activators (Gagnon, Bergeron et al.

2013, Gagnon, Bergeron et al. 2017) may decrease side effects by reducing the dosage of each compound.

Translational value of an early-in-life intervention

As highlighted above, taking all the possible pitfalls and risks of an early pharmacological intervention, including the vulnerability of the developing brain into consideration, timely interventions should be under scrutiny before they are transferred into clinical practice. On the other hand, the recognition of the potentials for correcting the trajectory of brain development makes this a very exciting time in the field of neurodevelopmental disorder research (Antonarakis, Skotko et al. 2020). This is even more relevant for DS research. Indeed, in the last five decades it is highly indicated that pregnant women at advanced maternal age (>35 years old) undergo prenatal screening for genetic disorders in the fetus with risk estimation for Down syndrome being the most successful among different screening concepts (Neagos, Cretu et al. 2011, Cuckle and Maymon 2016). In 2011, non-invasive prenatal testing using cell-free DNA (cfDNA) extracted from the maternal plasma became commercially available in clinical practice offering a safe and accurate diagnostic screening of the fetus (Carlson and Vora 2017, Suciu, Toader et al. 2019, Bull 2020). This preclinical screening can detect trisomy with 99.7% percent accuracy (Bull 2020). The possibility to diagnose DS during the first trimester of gestation opens a window of opportunity for planning of early therapeutic interventions. Conversely, other NDs such as ASD do not have biomarkers to be diagnosed earlier than the manifestation of the deficits mostly around the second year of life. Timely and differential diagnosis of subclinical pathological signs as early as possible is vital to prevent a vicious reactive plasticity cycle that give rise to the characteristic deficits of each ND (Ben-Ari 2017). Our data on hyperactivity, susceptibility to epileptic seizures and cognitive performance rescue after timed interventions indeed point to the value of early interventions to obtain long-lasting outcomes.

Despite NDs are caused by impaired brain developmental processes, treatments in adult life in animal models of NDs have proved also effective (Ehninger, Li et al. 2008, Castren, Elgersma et al. 2012). Nevertheless, interventions in animal models of NDs require chronic administration of the therapeutic intervention for the rescue of the deficits, as drug withdrawal experiments reveal

(Braudeau, Delatour et al. 2011, Dansie, Phommahaxay et al. 2013, Gantois, Pop et al. 2013, Deidda, Parrini et al. 2015, Pinto, Morelli et al. 2020). This necessity for continuous treatment administration is highlighted also by clinical trials, where they employ chronic treatments for the rescue of ND deficits (Lemonnier, Degrez et al. 2012, Grandgeorge, Lemonnier et al. 2014, Hadjikhani, Zurcher et al. 2015, Berry-Kravis, Hagerman et al. 2017).

The high translational value of a timed therapeutic intervention during a developmental window to rescue pathological developmental trajectories in their core could avoid the adverse effects following chronic treatment. In the case of bumetanide lifelong treatment on a daily basis in adulthood would have a strong impact on the life of treated individuals as bumetanide is a strong diuretic drug causing excessive diuresis and osmotic fluid imbalance (Konopacka, Qiu et al. 2015, Savardi, Borgogno et al. 2020). This is particularly inconvenient in the case of DS because the life expectancy of DS individuals has dramatically increased in the recent decades. Indeed, improvements in medical care has resulted in an increase of life expectancy from a mean age of 26 years and 4 years (median) in 1950 to average of 53 years and 58 years (median) in 2010 in the US (de Graaf, Buckley et al. 2017). Moreover, a lifelong drug administration is a commitment and includes several risks (i.e. drug resistance, drug-drug interactions). Our data suggests that treatment administered at the right moment during an opportunity window is a promising approach to treat or even prevent the emergence of a structural and functional deficit with lasting effects. One could speculate that this, in humans, could lead to the fact that you will not use any treatment as adults or less doses, nevertheless easing the burden of the side effects.

Finally, our study demonstrated gender-selective differences between male and female Ts65Dn animals and gender-selective impact of ELS in WT animals later in life. Other studies usually employ male animals to investigate pharmacotherapies and cognition in DS (Kleschevnikov, Belichenko et al. 2012, Deidda, Parrini et al. 2015, Savardi, Borgogno et al. 2020). To provide a more comprehensive insight of the effect of trisomy and the potential interventions future studies should include both males and females animals (Shaw, Klein et al. 2020). Of note, DS people studies have reported gender differences in behavioral and emotional impairments, with males being at a higher risk of more severe forms of intellectual disability, speech production deficits, thought and attention difficulties, and aggressiveness than DS females (Maatta, Tervo-Maatta et al. 2006, van Gameren-Oosterom, Fekkes et al. 2013). However, other studies do not find these

gender differences or report female-selective behavioral deficits, such as psychosis (Jacola, Hickey et al. 2014, Dykens, Shah et al. 2015). Further studies should investigate more extensively gender differences in the animal models of DS and DS people, which could help in the design of more efficient therapeutic interventions that can be transferred into the clinical practice.

Concluding remarks

In our study, we demonstrated that there is an opportunity window for long-term correction of learning and memory deficits, hyperactivity and susceptibility to seizures in the adult Ts65Dn mice. Moreover, our results suggest that an early intervention targeting NKCC1 by AAV-mediated knock down or by a commonly used, FDA drug is a promising approach for DS, although this approach should be under rigorous scrutiny for the possible pitfalls and risks following interventions of the vulnerable developing brain.

Methods

Animals

All animal procedures were approved by IIT licensing in compliance with the Italian Ministry of Health (D.Lgs 26/2014) and EU guidelines (Directive 2010/63/EU). A veterinarian was employed to maintain the health and comfort of the animals. Mice were housed in filtered cages in a temperature-controlled room with a 12:12 h dark/light cycle and with *ad libitum* access to water and food. All efforts were made to minimize animal suffering and use the lowest possible number of animals required to produce statistical relevant results, according to the ‘‘3Rs concept.’’ In this study, we used Ts65Dn mice maintained in their original genetic background (Reeves, Irving et al. 1995) by crossing (more than 40 times) Ts65Dn female to C57BL/6JEi x C3SnHeSnJ (B6EiC3) F1 males (Jackson Laboratories). Ts65Dn mice were genotyped by PCR as previously described (Duchon, Raveau et al. 2011, Reinholdt, Ding et al. 2011).

Animals aged between 12 and 16 weeks were used for behavioral experiments. Animals between 10-12 weeks were used for electrophysiology experiments. Both males and females were used for behavioral experiment, biochemistry, immunohistochemistry and electrophysiology experiments. Different cohorts of mice were used for the diverse tests. Ts65Dn and WT littermates were randomly assigned to bumetanide (Sigma; 0.2 mg/kg body weight) or vehicle groups (2% DMSO in saline) and treated twice a day, because of the pharmacokinetics of bumetanide (Cleary, Sun et al. 2013), by subcutaneous (SQ) injections from P2 to P15. Mice body weight was monitored twice a day during the treatment period and at P28, P60 and P90.

Behavioral Testing

Ts65Dn male and female mice (9–14 weeks old) were tested after bumetanide (0.2 mg/kg, SQ) or vehicle (2% DMSO in saline) treatment as pups from P2 until P15, or after bilateral intraventricular infection with a neuron-specific AAV9 construct with control amiR or NKCC1 amiR at P2. The battery of tests was run over a total period of 1 month (P90-P120) (seven behavioral tests for Ts65Dn and WT littermates in the following order: Startle acoustic reactivity, NOR, T-maze, NOL, Locomotor activity, CFC, Audiogenic seizures. The tasks (NOR, T-maze, NOL, CFC, Audiogenic seizures) were video-recorded and then analyzed manually by a blind operator.

Vehicle- and bumetanide-treated mice were always evaluated in parallel and with the same time schedule. In order to avoid any confounding effects, tests were administrated only once to individual mice. A detailed outline of the order of the tests for the different experimental cohorts is reported in Supplementary Table 1. After each trial or experiment, the diverse apparatus and objects were cleaned with 70% ethanol.

NOR Test

The test evaluates the spatial memory by measuring the ability of mice to recognize the new location of a familiar object. The NOR test was conducted in a gray acrylic arena (44 × 44 cm), evenly illuminated by overhead red lighting (12–14 lux). On day 1, mice were habituated to the arena by freely exploring the chamber for 15 min. On day 2, during the acquisition phase, mice were free to explore three different objects (different in color, size, shape, material) for 15 min. After 24 h, one object from the acquisition phase was replaced with a novel object, and the mice were tested for 15 min for their ability to recognize the new object. The time spent exploring each object was defined as the number of seconds during which mice showed investigative behavior (i.e., head orientation, sniffing occurring within < 1.0 cm) or clear contact between the object and the nose. The time spent exploring each object, expressed as a percentage of the total exploration time, was measured for each trial. The discrimination index was calculated as the difference between the percentages of time spent investigating the novel object and investigating the familiar objects: [discrimination index = (novel object exploration time/total exploration time × 100) - (familiar object exploration time/total exploration time × 100)]. As a control, we monitored object preference during the acquisition phase and exploration time in the acquisition phase and trial phase.

Object Location Test (OL)

The test evaluates the spatial memory by measuring the ability of mice to recognize the new location of a familiar object. The test was performed in a gray acrylic arena (44 × 44 cm), evenly illuminated by overhead red lighting (12–14 lux). Mice were first habituated to the chamber for 15 min on day 1. On day 2, during the acquisition phase, mice were exposed to two identical objects for 15 min. After 24 h, one of the two objects was moved during the test session to a novel location,

and the mice were tested for 15 min for their ability to recognize the new location of the object. The time spent exploring each object was defined as the number of seconds during which mice showed investigative behavior (i.e., head orientation, sniffing occurring within < 1.0 cm) or clear contact between the nose and the object. A discrimination index was calculated as the percentage of time spent investigating the object in the new location minus the percentage of time spent investigating the object in the old location [discrimination index = (new object location exploration time/total exploration time × 100) - (old object location exploration time/total exploration time × 100)]. As a control, we monitored object preference during the acquisition phase and the exploration time in the acquisition phase and trial phase.

T-Maze

The tests evaluates the short-term memory by measuring exploratory behavior of a new environment in mice. The T-maze is a black opaque plastic apparatus with a starting arm and two perpendicular goal arms, each equipped with a sliding door and evenly illuminated by overhead red lighting (12–14 lux). The T-maze test (spontaneous alteration protocol, 11 trials) evaluates short-term memory by analyzing the correct choice of the unexplored arm. The test was performed in similar way to that previously conducted on Ts65Dn mice (Kleschevnikov, Belichenko et al. 2012, Savardi, Borgogno et al. 2020). In each trial, a mouse was first placed in the starting chamber for 20 s. Then, the sliding door was removed, and the animal was free to explore the apparatus. When the mouse entered (with all four limbs) one of the two goal arms, the opposite arm was closed with the sliding door. When the mouse (free to explore the remaining part of the apparatus) returned to the starting area, the previously closed goal arm was opened. The trial was repeated 11 times. Entry into a goal arm opposite the one previously chosen was considered a correct choice, while entry into the previously explored arm was considered an incorrect choice. Alternation score was calculated as the percentage of correct choices (i.e., left-right or right-left) over the total number of the ten possible alternations.

CFC Test

The test evaluates the long-term associative memory by measuring the freezing time of the animals placed in a location where they had received an adverse stimulus (electric shock) 24 h earlier. The experiments were performed in a fear-conditioning system (TSE), which is a transparent acrylic conditioning chamber (23 3 23 cm) equipped with a stainless-steel grid floor. Mice were placed outside the experimental room in their home cages before the test and individually transported to the TSE apparatus in standard cages. Mice were placed in the conditioning chamber, and they received one electric shock (2 s, 0.75mA constant electric current) through the floor grid 3 min later. Mice were removed 15 s after the shock. After 24 h, mice were placed in the same chamber for 3 min. After 2 h, they were moved to a new context (black chamber with plastic gray floor and vanilla odor). The time spent frozen was scored and expressed as percentage of the total time analyzed.

Locomotor activity

Spontaneous locomotor activity was evaluated as previously described (Reeves, Irving et al. 1995) over a 24-h period (12/12 h dark/light cycle) during the fourth week of treatment. Horizontal activity, vertical and stereotyped movements were automatically evaluated using a VersaMax apparatus (AccuScan Instruments) equipped with an array of photocell beams. On the day of testing, mice were administered either vehicle or bumetanide just before the onset of the light phase (8 a.m.) and dark phase (8 p.m.) of the day.

Audiogenic seizures

Assessment of AGS sensitivity was conducted essentially as previously described (Westmark, Westmark et al. 2010, Westmark, Westmark et al. 2011) during the last (fourth) week of behavioral testing, as the last behavioral test. On the day of testing, mice were injected with bumetanide or vehicle 30 min before being introduced to a gridded cylindrical box (15-cm diameter) located in a sound-attenuating cubicle equipped with two loudspeakers and a video camera (TSE Systems). Mice were then exposed for 3 min to a 120-dB white noise and monitored for seizure induction. AGS severity was scored from 0 to 3 as previously described 64: 0, no response; 1, wild running; 2, clonic seizure; and 3, tonic seizure.

Acoustic startle response

The acoustic startle response (ASR) test assesses the reactivity to a sudden acoustic stimulus (usually a loud sound). It is a standardized test for an evolutionary conserved reflex across mammals (Koch 1999). Before the start of the behavioral battery (between P85-P90) each mice was placed in a plastic open cage. A device producing a sudden sound (20 kHz, 90 dB at 30cm) was used. Each mouse performed three trials (three sounds of the same intensity with 5s interval) and the score was averaged.

For behavioral experiments, we adopted the following exclusion criteria independent of genotype or treatment (before blind code was broken). In the T-maze test, we excluded mice that did not conclude the 10 trials within 20 min of the test. In the CFC test, we excluded mice showing very high non-associative freezing in the new context. This was defined as more than 30 s freezing during the 3-min test. In the OL and NOR test, we excluded animals showing very low explorative behavior. This was defined as less than 10 s of direct object exploration during the 15-min test. Following these criteria, a total of 11 mice among the NOR, NOL, CFC and T-maze tests were excluded.

Electrophysiological recordings

Hippocampal slices preparation

Animals between 10 and 16 weeks of age were used in the experiments. Both male and female mice were used in the electrophysiology experiments. Slices were obtained as previously described (Panuccio, Colombi et al. 2018). Briefly, mice were anesthetized with isoflurane and transcardially perfused with an ice-cold cutting solution (pH 7.4, oxygenated with 95% O_2 and 5% CO_2; see appendix A for the composition of the solutions). The animals were decapitated, and their brains were removed and immersed in ice-cold cutting solution. Combined entorhinal/hippocampal slices (400 μm thick, cut with a VT1000S Leica Microsystems vibratome) were incubated at 32 °C for 20 min in artificial cerebrospinal fluid (ACSF) oxygenated with 95% O_2 and 5% CO_2 (see appendix A for the compositions of the solutions). The slices were then maintained in ACSF solution for at least 1 hour to allow recovery.

Experimental protocol

Slices were pre-incubated with standard ACSF for 45 minutes. We adapted slices for 20 minutes on the MEA setup while continuously perfusing them with oxygenated ACSF and then recorded basal spontaneous activity for 30 minutes. We applied GABA (100 uM) or Bicuculline (20 uM) for the same amounts of time. We discarded the recordings made during the first 15 minutes of drug application to ensure complete exchange of the solution.

Data analysis

Data analysis was performed off-line using the custom software package SPYCODE (Bologna, Pasquale et al. 2010). The steps in the analysis are described briefly below. Raw traces were high-pass-filtered (>300 Hz) to isolate spikes from the low fluctuation of the signal (LFP). We computed the spike detection as previously described (Maccione, Gandolfo et al. 2009). Briefly, the method used three parameters: (1) a differential threshold (DT) set independently for each channel and computed as 8-fold the standard deviation (SD) of the noise of the signal; (2) a peak lifetime period (PLP) set to 2 ms; (3) a refractory period set to 1 ms.

The algorithm scans the raw data to discriminate the relative minimum or maximum points. Once a relative minimum point is found, the nearest maximum point is searched within the following PLP window (or vice versa). If the difference between the two points is larger than DT, a spike is identified and its timestamp saved. Then, to characterize the activity level of the CA1 region, we computed the mean firing rate (MFR), which is defined as the mean number of spikes per second, computed over the total recording time. We considered active electrodes as those presenting a firing rate higher than 0.02 spikes per second.

We computed the mean firing ratio (MFR), which is defined as the mean firing rate after drug administration (either GABA or Bicuculline) over the mean firing rate during baseline. Values equal to 1 indicate no change in MFR after drug application in the bath. Values above 1 indicated an increase while values below 1 a decrease in MFR after drug application.

$$MFR\ ratio = \frac{MFR\ GABA/Bic}{MFR\ basal}$$

We computed the percentage of variation for each active electrode and we evaluated significant changes with respect to the basal condition. The percentage of variation was computed as follows:

$$\%\, Variation\ Ch = \frac{MFRdrug - MFRbasal}{MFRbasal}$$

We considered significant those electrodes showing a percentage of variation up to 10% with respect to the basal condition.

Biochemistry

Protein extraction

For total protein extraction, hippocampal samples were homogenized in RIPA buffer (1% NP40, 0.5% deoxycholic acid, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 50 mM Tris, pH 7.4) containing 1 mM PMSF, 10 mM NaF, 2 mM sodium orthovanadate and 1% (v/v) protease and phosphatase inhibitor cocktail (Sigma). The samples were clarified by centrifugation at 20,000g, and the protein concentration was determined using a Bicinchoninic Acid Assay (BCA) kit (Pierce).

Western blotting

For immunoblot analysis, equal amounts of protein were run on 4–12% Bis-Tris NuPAGE (Invitrogen) or Criterion-XT (Bio-Rad) gels and transferred onto nitrocellulose membranes (GE Healthcare). Membranes were probed with mouse anti-NKCC1 (clone T4, Developmental Studies Hybridoma Bank; 1:4,000), rabbit anti-KCC2 (Millipore, catalog no. 07-432; 1:4,000), rabbit anti β-actin (Sigma, catalog no. A2066; 1:10,000), mouse anti-APP (clone 22C11, Millipore, catalog no. MAB248; 1:2,000), followed by HRP-conjugated secondary antibodies goat anti-rabbit and goat anti-mouse (ThermoFisher Scientific, catalog nos. 31460 and 31430, respectively; 1:5,000). Membranes were developed with SuperSignal West Pico chemiluminescent substrate (ThermoFisher Scientific). The chemiluminescent signals were acquired on iBright CL1500 Imaging System (ThermoFisher Scientific), and the band intensities were quantified using

ImageQuant software (GE Healthcare). In some experiments, membranes were stripped and reprobed with a second antibody.

Immunohistochemistry

Animals were deeply anesthetized and transcardially perfused with 4% paraformaldehyde in 100 mM phosphate buffer (PB), pH 7.4. Brains were collected, post-fixed for 24 h in the same fixative solution, cryo-preserved in 30% sucrose in PB and stored at -80 °C until use. Immunohistochemistry was performed on 30-µm coronal serial sections to visualize distribution of the GFP expression by the AAV9 construct. The sections were stained with the nuclear dye Hoechst-33342 (Sigma-Aldrich). Fluorescence images were captured with Neurolucida (Mbf Bioscience) microscope equipped with a 10X air objective and a motorized stage. For each section, serial images were acquired to construct a contouring of the whole slice. Reconstruction of the images acquired from serial section were put together using ImageJ software (http://imagej.nih.gov/ij/).

Viral preparations

AAV serotype 9 (AAV9) used in this study were produced using a slight modification of the adenovirus-free transient transfection methods described before (Matsushita, Elliger et al. 1998, Ayuso, Mingozzi et al. 2010). Briefly, adherent HEK293 cells grown in roller bottles were transfected with three plasmids containing the adenovirus helper proteins, the AAV Rep and Cap genes, and the ITR-flanked transgene expression cassette. Three days after transfection, cells were harvested, lysed by sonication, and treated with benzonase (Merck-Millipore). Vectors were purified using two successive ultracentrifugation rounds in cesium chloride density gradients. Full capsids were collected. The final product was formulated in sterile phosphate buffered saline containing 0.001% of pluronic F-68 (Sigma), and stored at -80°C. Titers of the AAV vector stocks were determined by SDS-PAGE followed by SYPRO Ruby protein gel stain and band densitometry. Bilateral intraventricular injection of the viral construct (control amiR or NKCC1

amiR) were performed in pups at P2. Dose injected: $1*10^{10}$ vg/hemisphere resulting in a dose of $2*10^{10}$ vg/mice.

Statistical Analysis

The results are presented as the means ± SEM. The statistical analysis was performed using SigmaPlot (Systat) and GraphPad (Prism) software. Where appropriate, the statistical significance was assessed using the following parametric test: two-tailed unpaired t-test, one-way ANOVA followed by Dunnet post hoc test, two-way ANOVA or two-way ANOVA on ranks followed by all pairwise Tukey post hoc test, Holm–Sidak post hoc test or Student-Newman-Keuls Method. Chi Square test with Sidak adjustment for multiple comparisons was used for AGS analysis. P-values < 0.05 were considered significant. Outliers were excluded only from the final pool of data by a Grubb's test run iteratively until no outliers were found. For the analysis of the weight of the treated animals we carried out linear analyses of variance (ANOVA) using the 'ezANOVA' function of the R software (Michael Lawrence, 2016). 'Genotype' and 'Treatment' were considered as between factors and 'Postnatal day' as within factor. P-values were Greenhouse-Geisser adjusted when sphericity assumptions were violated. Post hoc t-tests were corrected with Bonferroni method.

Supplementary Tables

Supplementary table 1

Mice cohorts for behavioral testing after infection with AAV9 control or NKCC1 amiR as pups

Cohort #	Animals	NOR	OL	T-maze	CFC	Locomotor activity	Audiogenic seizures
1	WT control amiR: 6 Ts65Dn control amiR: 2 WT NKCC1 amiR: 13 Ts65Dn NKCC1 amiR: 2	✓	✓	✓	✓		
2	WT control amiR: 6 Ts65Dn control amiR: 4 WT NKCC1 amiR: 12 Ts65Dn NKCC1 amiR: 7	✓	✓	✓	✓		
3	WT control amiR: 14 Ts65Dn control amiR: 4 WT NKCC1 amiR: 2 Ts65Dn NKCC1 amiR: 2	✓	✓	✓	✓		
4	WT control amiR: 0 Ts65Dn control amiR: 0 WT NKCC1 amiR: 8 Ts65Dn NKCC1 amiR: 3					✓	✓
5	WT control amiR: 7 Ts65Dn control amiR: 2 WT NKCC1 amiR: 0 Ts65Dn NKCC1 amiR: 0					✓	✓
6	WT control amiR: 0 Ts65Dn control amiR: 0 WT NKCC1 amiR: 5 Ts65Dn NKCC1 amiR: 3						✓
7	WT control amiR: 6 Ts65Dn control amiR: 4 WT NKCC1 amiR: 0 Ts65Dn NKCC1 amiR: 0						✓
8	WT control amiR: 0 Ts65Dn control amiR: 0 WT NKCC1 amiR: 10 Ts65Dn NKCC1 amiR: 4					✓	
9	WT control amiR: 0 Ts65Dn control amiR: 0 WT NKCC1 amiR: 3 Ts65Dn NKCC1 amiR: 3					✓	
10	WT control amiR: 2 Ts65Dn control amiR: 5 WT NKCC1 amiR: 0 Ts65Dn NKCC1 amiR: 0					✓	✓

Supplementary table 2

Mice cohorts for behavioral testing after vehicle and bumetanide treatment as pups

Cohort #	Animals	NOR	OL	T-maze	CFC	Locomotor activity	Audiogenic seizures	Startle
1	WT vehicle: 3 Ts65Dn vehicle: 1 WT bumetanide: 4 Ts65Dn bumetanide: 6	✓	✓	✓	✓		✓	✓
2	WT vehicle: 6 Ts65Dn vehicle: 3 WT bumetanide: 7 Ts65Dn bumetanide: 8	✓	✓	✓	✓		✓	✓
3	WT vehicle: 5 Ts65Dn vehicle: 4 WT bumetanide: 0 Ts65Dn bumetanide: 0	✓	✓	✓	✓		✓	✓
4	WT vehicle: 9 Ts65Dn vehicle: 4 WT bumetanide: 6 Ts65Dn bumetanide: 3	✓	✓	✓	✓	✓	✓	✓
5	WT vehicle: 3 Ts65Dn vehicle: 1 WT bumetanide: 4 Ts65Dn bumetanide: 1	✓		✓	✓			✓
6	WT vehicle: 5 Ts65Dn vehicle: 5 WT bumetanide: 6 Ts65Dn bumetanide: 3		✓	✓				✓
7	WT vehicle: 0 Ts65Dn vehicle: 3 WT bumetanide: 3 Ts65Dn bumetanide: 1					✓		✓
8	WT vehicle: 11 Ts65Dn vehicle: 5 WT bumetanide: 2 Ts65Dn bumetanide: 2					✓		✓
9	WT vehicle: 0 Ts65Dn vehicle: 0 WT bumetanide: 8 Ts65Dn bumetanide: 4					✓		✓

Supplementary table 3

Mice cohorts for MEA recordings after infection with AAV9 control or NKCC1 amiR as pups

GABA

Groups	# Slices	# Mice
WT control amiR	6	6
Ts65Dn control amiR	10	9
WT NKCC1 amiR	13	9
Ts65Dn NKCC1 amiR	7	6

BICUCULLINE

Groups	# Slices	# Mice
WT control amiR	7	7
Ts65Dn control amiR	8	7
WT NKCC1 amiR	9	10
Ts65Dn NKCC1 amiR	8	8

Supplementary table 4

Mice cohorts for MEA recordings after vehicle and bumetanide treatment as pups

GABA

Groups	# Slices	# Mice
WT vehicle	6	2
Ts65Dn vehicle	8	3
WT bumetanide	8	3
Ts65Dn bumetanide	12	5

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Appendix

Chloride transporters in physiological brain development and neurodevelopmental disorders: The case of the Down syndrome

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CHAPTER 21

Chloride transporters in physiological brain development and neurodevelopmental disorders: The case of the Down syndrome

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Introduction

Many philosophers and scientists initially defined human cognitive development by describing changes in an individual's social and personal contexts rather than focusing on the biological processes of brain development. It was not until the era of Camillo Golgi and Santiago Ramon y Cajal (19–20th centuries) that the foundations of modern neuroscience were set and that the concept of neurodevelopment evolved. Many advances have been made since then, thereby elucidating the different biological mechanisms underlying behavior in both physiological and pathological conditions. Nevertheless, many more studies are necessary to adequately bridge neuronal physiological properties and biological rules for complex behaviors. Here, we focus on how the neuronal cation chloride cotransporters (CCCs), by regulating GABAergic transmission, play fundamental roles in determining proper neural development. Moreover, we describe how impairments of the expression and function of these CCCs can lead to the onset of defective brain development, which underlies aberrant behaviors in several psychiatric/neurological conditions also referred to as neurodevelopmental disorders.

Brain development and the role of GABA

Three distinct yet partially overlapping phases are involved in the establishment of neuronal circuits during development: an early, innate activity-independent phase, a later phase driven by spontaneous patterns of neuronal activity, and a final phase dependent on neuronal activity driven by sensory experience.

Neuronal Chloride Transporters in Health and Disease. https://doi.org/10.1016/B978-0-12-815318-5.00021-2

During the first two phases, neuronal progenitors proliferate and differentiate, and newly born neurons mature morphologically and migrate to their final locations in the brain, establishing a first set of neuronal connections (Ben-Ari, 2001; Spitzer, 2006). After the development of sensory organs, the final phase starts, and neuronal activity driven by sensory experience from the external environment refines the initial neuronal circuitry (Feller, 1999; Hadders-Algra, 2018; Leighton and Lohmann, 2016).

GABA (γ-aminobutyric acid) is the first neurotransmitter to be functional in developing neuronal networks and it plays major roles in all three phases of brain development. GABA is synthesized in the CNS from the L-glutamic acid by the enzyme L-glutamic decarboxylase (GAD), which is present in two isoforms, GAD65 and GAD67 (Buddhala et al., 2009). GABA exerts its action by binding two different types of receptors: the ionotropic $GABA_A$ receptor ($GABA_AR$) and the metabotropic $GABA_B$ receptor ($GABA_BR$). $GABA_A$Rs are ligand-gated ion channels formed by the assembly of 5 different subunits out of a total of nineteen potential $GABA_AR$ subunits (α1-6, β1-3, γ1-3, δ, ε, θ, π, ρ1-3). Different combinations of five subunits confer $GABA_A$Rs' diverse properties with respect to ionic gating dynamics, cellular localization, and physiological functions (Has and Chebib, 2018; Koduvayur et al., 2014). $GABA_A$Rs are mostly permeable to chloride (Cl^-) and they mediate slow and tonic extra-synaptic currents or fast and phasic synaptic currents, depending on the presence of the different combination of the receptor subunits. Tonic currents are mediated by low concentrations of ambient GABA that escaped from the synaptic cleft and can activate subtypes of extrasynaptic $GABA_A$ receptors with high affinity (Brickley et al., 1999; Cellot and Cherubini, 2013; Farrant and Nusser, 2005). Conversely, the phasic GABAergic inhibition occurs at the postsynaptic site of the cleft, when GABA is released at a high concentration from the presynaptic vesicles. During early brain development, before the onset of GABAergic synaptic activity, extrasynaptic tonic current plays a major role in mediating network activity (Brickley et al., 1999; Farrant and Nusser, 2005; Kilb et al., 2013).

For both tonic and phasic GABAergic currents, Cl^- can flow through the $GABA_AR$ in both the directions, depending on its concentration gradient across the cell membrane and the membrane resting potential of the neuron. In the adult CNS under physiological conditions, there is a low Cl^- concentration inside the neurons. Thus, the direction of Cl^- flow is inward, and GABA exerts hyperpolarizing and inhibitory actions (Kahle et al., 2013). Conversely, during early neurodevelopment, there is a high Cl^- concentration inside the cell. Thus, opening of the $GABA_AR$ causes a chloride efflux from the cell and GABA depolarizes the membrane (Kahle et al., 2013). This depolarization leads to activation of voltage-gated calcium channels and removal of the Mg^{2+} block from NMDA receptors, causing further membrane depolarization and calcium influx into the cell. This is vital for the activation of second messengers, including

calcium sensitive kinase (PKC) and calcium/calmodulin-dependent protein kinase II (CamKII), that participate in neuronal migration, differentiation and synaptogenesis (Ben-Ari, 2002, 2014; Cellot and Cherubini, 2013; Leinekugel et al., 1997; Takayama and Inoue, 2010).

Later in development, when the initial set of connections between developing neurons is built, depolarizing GABA also controls spontaneous neuronal activity in the form of network action potential bursts of different durations and intervals across multiple brain areas. Interestingly, these bursts of spontaneous neuronal activity during early development have been described in different species including the rodent, chick, turtle, ferret and rabbit (Aguado et al., 2003; Ben-Ari, 2001; Wong, 1999). Moreover, evidence exists that in primate fetuses there is a complex hippocampal network capable of generating spontaneous and paroxysmal synchronized activities in utero (Khazipov et al., 2001). Interestingly, early patterns of neuronal synchronized activity have also been described in humans (preterm babies) by EEG and fMRI studies (Arichi et al., 2017; Khazipov and Luhmann, 2006; Tolonen et al., 2007). Nevertheless, the contribution of depolarizing GABA during these early patterns of neuronal activity in humans has not been investigated yet. The only data available in humans, derive from neurons differentiated from human pluripotent stem cells in vitro, in which early synchronous network activity is based mainly on gap junctions and emerges when the strong depolarizing GABA activity decrease (Makinen et al., 2018).

Notably, when GABA switches its action from depolarizing and mostly excitatory to hyperpolarizing and inhibitory later in life by upregulating KCC2 gene expression, the neuronal network activity oscillations disappear (Leitch et al., 2005; Sernagor et al., 2003). Interestingly, for at least some brain structures, this phenomenon coincides with the development of complex behavior related to that specific brain structure. For example, the striatum completes its immature activity patterns precisely when pups begin coordinated locomotor behavior (Dehorter et al., 2012).

Finally, proper GABAergic transmission is fundamental during critical periods of enhanced neuronal connectivity and plasticity driven by sensory system experiences deriving from individuals' own life experiences (Begum and Sng, 2017; Berardi et al., 2000; Hensch, 2004; Hensch and Fagiolini, 2005; Hensch and Quinlan, 2018; Sommeijer et al., 2017; Takesian and Hensch, 2013; Zhang et al., 2018).

Chloride transporters in physiological brain development

The understanding of the mechanisms regulating intracellular Cl^- concentration during development has gained a lot of attention in recent years because of the

fundamental role of Cl^- in modulating GABAergic transmission and its consequent implication in neurodevelopmental processes. In neurons, the main regulators of Cl^- homeostasis are the CCCs (Blaesse et al., 2009; Li et al., 2002), especially the sodium-potassium-chloride cotransporter isoform 1 (NKCC1) and the potassium-chloride symporter isoform 2 (KCC2). NKCC1 is highly expressed in immature neurons during development, as it transports Cl^- inside the cells, leading to a high intracellular Cl^- concentration (~30 mM), and depolarizing and mostly excitatory GABA actions (Achilles et al., 2007; Dzhala et al., 2005). On the other hand, KCC2 is highly expressed in mature neurons, where it keeps the intracellular Cl^- level at low values (4–6 mM; Delpire and Kahle, 2017), thereby determining the hyperpolarizing and inhibitory GABA action (Ben-Ari, 2002, 2014; Rivera et al., 1999; Stein et al., 2004).

Interestingly, this mature pattern of low NKCC1 and high KCC2 expression develops earlier in the evolutionarily older structures (e.g., spinal cord, brainstem, hypothalamus; Watanabe and Fukuda, 2015). Indeed, NKCC1 and KCC2 are highly expressed in the mouse spinal cord from E11.5-E13.5 (Delpy et al., 2008) and starting from E15.5, NKCC1 decreases its expression, while KCC2 remains highly expressed. This difference generates the depolarizing to hyperpolarizing GABA switch in motor neurons around E17.5 (Branchereau et al., 2002). Notably, recordings at E18.5 in $KCC2^{-/-}$ mice showed that motorneurons exhibited GABA and glycine (that also binds to Cl^--permeable ionic receptors) excitation, thus highlighting KCC2 importance in determining the polarity and efficacy of GABAergic inhibitory transmission (Hubner et al., 2001).

In the hypothalamus, the presence of NKCC1 mRNA expression was not clear in rodent embryos, and its expression was weak postnatally. Conversely, at E14.5, KCC2 mRNA is strongly present and maintains its expression into adulthood in rodents (Li et al., 2002; Wang et al., 2002). This suggests that the developing hypothalamus presents a mature-like phenotype characterized by GABAergic inhibitory activity. Nevertheless, electrophysiological recordings in developing hypothalamic neurons showed that GABA exerts a depolarizing and excitatory action (Gao and van den Pol, 2001). This seemingly contrasting results could be explained by posttranscriptional mechanisms like phosphorylation able to modulate the activity of the two CCCs, sex-specific and/or cell-specific expression of KCC2 (Watanabe and Fukuda, 2015).

In the thalamus, NKCC1 mRNA is not present during the embryonic stages in rats (Wang et al., 2002). Conversely, KCC2 mRNA is already found at E12 in rodents thalamus, when the region begins forming, with the exception of the dorsomedial part, which expresses KCC2 later at E18 (Li et al., 2002; Wang et al., 2002; Watanabe and Fukuda, 2015). Notably, NKCC1 mRNA is stably expressed in the thalamic tissue postnatally in rats (from soon after birth into adult life), suggesting a possible low hyperpolarizing or even depolarizing action of GABA in the adult thalamus (Wang et al., 2002; Watanabe and Fukuda, 2015).

In the rat cerebellum, NKCC1 mRNA was not detected in Purkinje cells at any postnatal age. Conversely, KCC2 mRNA was found at E15.5 in mouse Purkinje cells and at P1 in rat Purkinje cells when the cells begin differentiation (Mikawa et al., 2002). Interestingly, NKCC1 mRNA was observed in rats in the external granule layer, where the immature granule cells (later developing in comparison to Purkinje cells) are located at P7 and P14, and in the internal granular layer in postmigratory granule cells after P7 (Mikawa et al., 2002). Moreover, KCC2 transcripts were detectable already at P3 in mouse and at P7 in rat granule cells (Mikawa et al., 2002; Stein et al., 2004).

In the rat hippocampus, NKCC1 mRNA is strongly present in the neuroepithelium at E18 (Watanabe and Fukuda, 2015), peaking in the first postnatal week and then decreasing by P14-P15 (Pfeffer et al., 2009; Plotkin et al., 1997; Wang et al., 2002). Instead, KCC2 mRNA signals can be detected first in the CA3 region at E15.5 and in the CA1 region at E18.5 in mice; it then reaches adult levels at P15 (Stein et al., 2004). In the same study, western blot analysis showed a time course of KCC2 protein expression closely parallel to the detection of the KCC2 transcript described above (Stein et al., 2004).

In the neocortex, NKCC1 transcripts have been detected as early as E12.5 in scattered cells of the mouse neuroepithelium and in the ventricular zone (VZ) of the ganglionic eminences (Li et al., 2002; Watanabe and Fukuda, 2015) where the neuronal progenitors are located. By E14.5, NKCC1 mRNA is upregulated in the proliferative zones of the lateral and medial ganglionic eminence in mice (Watanabe and Fukuda, 2015). Then, NKCC1 expression in the VZ decreases in late mouse embryonic development (E17-P0; Caviness Jr. et al., 1995). In the differentiated cells of the cortical plate both mRNA and protein of NKCC1 are strongly present in rats (Li et al., 2002; Watanabe and Fukuda, 2015). Interestingly, KCC2 mRNA signals are not detected in the mouse neocortex until P0 (Li et al., 2002; Wang et al., 2002). Although most of the work on the presence or absence of the CCCs is based on mRNA evidence, some data on the protein expression are available for the rat neocortex. In particular, NKCC1 is highly expressed postnatally between P3-P14, whereas KCC2 is expressed at low levels during the first 2 weeks after birth and is upregulated by P21 (Dzhala et al., 2005).

Finally, NKCC1 is highly expressed both in the developing and adult choroid plexus in mice and rats (Kanaka et al., 2001; Li et al., 2002). There, NKCC1 is located on the apical membrane of epithelial cells and it plays a key role in the formation of the cerebrospinal fluid (CSF). Conversely, KCC2 is not expressed in the choroid plexus epithelial cells in mice (Steffensen et al., 2018).

The developmental expression profiles of NKCC1 and KCC2 have also been investigated in humans. At the mRNA level, both NKCC1 and KCC2 increased with gestational age during the second trimester and after birth in the prefrontal

cortex, while only KCC2 increased in the hippocampus across the second trimester, but both NKCC1 and KCC2 increased after birth (Hyde et al., 2011). A more recent study found lower levels of NKCC1 mRNA during the prenatal period (10 post conception week (PCW)-birth) with an increase in the postnatal age (birth-90 years) in the 16 brain areas analyzed (Sedmak et al., 2016). At the protein level, the expression of NKCC1 in the time window between PCW 31–41 was high with a peak at PCW 35 and decreased from the first year of life (PCW 54–92) to adulthood in the parietal cortex. KCC2 expression was low during the entire fetal and neonatal period (PCW 20–41) and increased over the first year of life (Dzhala et al., 2005). Nevertheless, other studies found a robust presence of both KCC2 mRNA and protein during the second half of gestation in the neocortex, indicating that high KCC2 expression starts prenatally (Kaila et al., 2014). Notably, expression of KCC2 was observed as early as PCW16 in a subset of subplate neurons (Bayatti et al., 2008). Moreover, KCC2-immunoreactive neurons were described in the hippocampus and entorhinal cortex as early as PCW 25 (which was the earliest age tested), and these neurons reach adult levels during the first six postnatal months (Dzhala et al., 2005; Sedmak et al., 2016). Interestingly, the overall ratio of NKCC1 to KCC2 is very high in pediatric human brains, and it decreases until approximately 2 years of life and then it remains at the adult levels (Jansen et al., 2010).

The role of NKCC1 and KCC2 in neuronal proliferation, migration, and network integration

The important role of NKCC1 and KCC2 in driving and regulating fundamental processes of proper brain development has been widely demonstrated in rodents by diverse experimental approaches ranging from knock out (KO) animals to modulating the expression and/or the activity of the two CCCs by pharmacological inhibition (e.g., with the widely used FDA-approved drugs bumetanide and furosemide), RNA interference, overexpression (Schulte et al., 2018).

NKCC1 plays a key role in cell proliferation and apoptosis

The role of NKCC1 in brain cell proliferation has been demonstrated by a number of studies. In particular, ex vivo investigations have shown that NKCC1 is expressed in radial glial cells in rats (progenitors of excitatory cortical neurons; Li et al., 2002; Noctor et al., 2001) in the cortex, although it is not in βIII-tubulin- (marker of postmitotic neurons) positive regions (Li et al., 2002). Interestingly, NKCC1 is highly expressed also in the ganglionic eminence, the brain region that gives birth to GABAergic interneurons (Li et al., 2002). In agreement with the abovementioned studies, NKCC1 knockdown mice have defects in the proliferation of neural precursor cells of the SVZ (Young et al., 2012) and in the proliferation of the neural progenitors of the

lateral ganglionic eminences (Magalhaes and Rivera, 2016). Moreover, pharmacological blocking of NKCC1 with bumetanide inhibits cell proliferation in neuronal precursors of the subventricular zone in mice (Sun et al., 2012). The role of NKCC1 in brain cell proliferation has been demonstrated also in vitro in mouse oligodendrocyte precursor cells, where NKCC1 inhibition is associated with attenuation in cell cycle progression (Fu et al., 2015).

Another fundamental stage where NKCC1 plays a role is programmed cell death during development. In particular, NKCC1 is implicated in the activity-regulated cell death in Cajal-Retzius neurons, a population that mostly disappears from apoptosis early in life in the mouse developing cortex (Blanquie et al., 2017). Pharmacological inhibition of NKCC1 by bumetanide in vitro or genetic deletion of the cotransporter in vivo (NKCC1$^{-/-}$ mice) rescued the population of Cajal-Retzius neurons from apoptosis (Blanquie et al., 2017).

NKCC1 and KCC2 regulate neuronal migration

The depolarizing GABA transmission by high expression of NKCC1 in the ventricular zone and cortical plate (Shimizu-Okabe et al., 2002) plays a role in the migration of newly generated rat cortical neurons (Behar et al., 1996, 1998; Heck et al., 2007). In particular, both knocking down of NKCC1 (shRNA) and pharmacological manipulations of NKCC1 by bumetanide in neuroblasts in mice from the rostral migratory stream in organotypic slice cultures reduced migratory speed without affecting the direction of the migration (Mejia-Gervacio et al., 2011). Although this piece of evidence points to a possible role for NKCC1 in physiological interneuron migration, a direct demonstration of the involvement of NKCC1 in cortical excitatory-neuron migration is still missing. Indeed, the knockdown of NKCC1 in vivo in mice during neurodevelopment through in utero electroporation resulted in a disruption of cortical neuron morphology, but unfortunately, neuronal migration was not evaluated in that same study (Wang and Kriegstein, 2008). Nevertheless, several lines of evidence suggest that altered expression of NKCC1 underlies cortical malformations and neuronal ectopy in pathological conditions (Fukuda and Wang, 2013; Koyama et al., 2012; Shimizu-Okabe et al., 2007). In particular, high expression of NKCC1 and low expression of KCC2 were found in cortical plate neurons involved in micro-gyral cortical malformations in rodents in vivo (Fukuda and Wang, 2013; Shimizu-Okabe et al., 2007). Moreover, either knockdown of NKCC1 by RNA interference or its pharmacological inhibition by bumetanide both rescued the migration deficits of granule cells in the dentate gyrus (DG) in a rat model of febrile seizures in vivo (Koyama et al., 2012). Furthermore, some evidence indicates a role for NKCC1 in the migration of glioma cells both in vitro and in vivo in mice (Haas and Sontheimer, 2010). While these in vivo studies

suggest that high NKCC1 expression could affect neural migration and pathological conditions, it is not clear whether physiological levels of NKCC1 regulate migration in vivo in nonpathological conditions.

As for NKCC1, some evidence exists that KCC2 also mediates migration of interneurons, where its upregulation works as a stop signal both in vitro and in vivo in mice and in organotipic cultures from a ferret model of cortical dysplasia (Abbah and Juliano, 2014; Bortone and Polleux, 2009; Inamura et al., 2012; Miyoshi and Fishell, 2011). On the other hand, KCC2 expression in excitatory neurons increases only after they have completed their migration across different cortical layers and other brain areas in rats (Cancedda et al., 2007). In agreement with these data, overexpression of KCC2 in newly born excitatory cortical neurons did not affect their migration in rats (Cancedda et al., 2007). Nevertheless, this may possibly be because high levels of taurine inhibit KCC2 function at embryonic stages, thus preserving the depolarizing GABA signaling (Inoue et al., 2012). Interestingly, a structural role for KCC2 in neural crest cell migration and early radial glia migration in mice (at E9.5) was reported to occur through the interaction with the cytoskeleton-associated protein 4.1N and independently of the ion-transport action (Horn et al., 2010).

NKCC1 and KCC2 regulate neuronal morphological maturation

Both NKCC1 and KCC2 play fundamental roles in neuronal branching and in the establishment, maintenance and plasticity of synapses (Kaila et al., 2014; Khalilov et al., 2011; Sedmak et al., 2016). In particular, a high Cl^- concentration maintained by the high expression of NKCC1 and the low expression of KCC2, is fundamental for neuronal morphological maturation.

For example, NKCC1 was observed in the tip of growing neurites (Nakajima et al., 2007), and both its knockdown by RNA interference (Nakajima et al., 2007, 2011a,b) or its pharmacological inhibition by treatment with bumetanide (Nakajima and Marunaka, 2016) abolished neurite outgrowth in vitro in PC12 cells. Accordingly, knockdown of NKCC1 in vivo disrupted the dendritic maturation of mouse cortical neurons (Wang and Kriegstein, 2008; Young et al., 2012). Moreover, NKCC1 activation is also required for neurite growth in injured rodent adult neurons in vivo (Modol et al., 2015; Pieraut et al., 2007, 2011). Furthermore, NKCC1 has been involved in the maturation of both rodent excitatory and inhibitory synapses (Nakanishi et al., 2007; Pfeffer et al., 2009; Wang and Kriegstein, 2008). Although $NKCC1^{-/-}$ mice do not exhibit morphological alteration of hippocampal dendritic arborization, they presented delayed maturation of GABAergic and glutamatergic synapses (Pfeffer et al., 2009). Accordingly, in utero NKCC1 knockdown in mouse excitatory cortical neurons affected the physiological development of excitatory and inhibitory synapses (Wang and Kriegstein, 2008). Moreover, pharmacological NKCC1 inhibition by bumetanide during mouse cortical development

disrupted AMPA synapse maturation, although it did not affect NMDA receptor signaling (Wang and Kriegstein, 2011). In line with studies on NKCC1 downregulation/inhibition, premature expression of KCC2 by in utero electroporation in a subpopulation of rat cortical neuron progenitors severely impacted morphological maturation, with fewer and shorter neurites (Cancedda et al., 2007); but see (Fiumelli et al., 2013) together with an increased number of dendritic spines (Fiumelli et al., 2013). This seeming discrepancy (decreased neurite complexity vs. increased dendritic spine density) could be possibly explained by considering the two different and specific roles of KCC2 in dendritic growth and in spine maintenance. In particular, in dendritic growth, which possibly requires GABA depolarizing signaling, low KCC2 expression could be needed to the maintenance of the proper chloride homeostasis. Conversely, KCC2 has been showed to exert a structural role in the process of dendritic spine formation, independently of its Cl^- transporter activity, through the interaction with cytoskeleton (Fiumelli et al., 2013). Interestingly, in a different study KCC2 has been demonstrated to interact with the submembrane actin cytoskeleton by binding to 4.1N protein (Li et al., 2007), which could represent the molecular mechanism underlying the spinogenesis. Moreover, different studies showed that KCC2 is highly expressed in spine head where AMPA and NMDA receptor are located, playing a role in maintenance of glutamatergic synapses (Blaesse and Schmidt, 2015; Chamma et al., 2012), in constraining lateral diffusion of AMPA receptors, and in regulating their content at the spine (Chevy et al., 2015; Gauvain et al., 2011; Llano et al., 2015). In agreement with previous studies, KCC2 knockout mice exhibit large alterations in synaptic and neuronal network activity in the CA3 region of the hippocampus (Khalilov et al., 2011).

Finally, premature KCC2 overexpression found in a rat model of atypical febrile seizures and in a variant of KCC2 found in an Australian family with febrile seizures cause a reduction in dendritic spine number (Awad et al., 2016; Puskarjov et al., 2014b) Interestingly, the reduction of the premature KCC2 expression rescued the alterations in spine density and morphology and the seizure susceptibility in the same rat model of febrile seizures (Awad et al., 2016).

The role of NKCC1 and KCC2 in the critical period of brain plasticity

The critical period for sensory system plasticity has been widely investigated in the visual system, starting from the pioneering studies of Hubel and Wiesel in the half of the 20th century. Interestingly, proper development of GABAergic transmission is crucial for both the opening and the closure of the critical period plasticity in the visual cortex. Indeed, manipulation of inhibition, by prematurely enhancing or reducing GABAergic signaling during development interferes with the onset of the rodent critical period plasticity (Fagiolini

et al., 2004; Fagiolini and Hensch, 2000; Huang et al., 1999; Iwai et al., 2003). Notably, reducing GABAergic activity in adult animals reopens the critical period in the visual cortex (Harauzov et al., 2010). More recently, Deidda and colleagues demonstrated that depolarizing GABA during early postnatal development plays a pivotal role in the duration of the critical period for visual cortical plasticity later in life. In particular, they observed that pharmacological inhibition of NKCC1 with bumetanide from P3 to P8 in rats extended the duration of the critical period into adulthood, with a mechanism dependent on the neurotrophin BDNF (Deidda et al., 2015a).

Expression and role of other NKCCs and KCCs in the developing brain

In addition to NKCC1, the NKCC family also contains NKCC2. NKCC2 is highly expressed in the apical membrane of the epithelial cells of the thick ascending limb in the kidney and the macula densa cells (specialized sensor cells detecting changes in the fluid composition of the distal tubule), where it facilitates the reabsorption of sodium and chloride ions into the blood (Delpire and Gagnon, 2018; Edwards et al., 2014). Moreover, NKCC2 is strongly expressed in the epithelial layer of the endolymphatic sac in humans, a part of the vestibular system (Kakigi et al., 2009). Notably, NKCC2 immunoreactivity is also present in vasopressinergic and oxytocinergic neurons in the hypothalamo-neurohypophyseal system in the rat brain (Konopacka et al., 2015). Blockade of NKCC2 leads to pronounced natriuresis, kaliuresis and diuresis (Becker et al., 2003; Castrop and Schiessl, 2014; Gamba and Friedman, 2009; Hannemann et al., 2009; Schiessl and Castrop, 2015). Loss of function mutations of the gene coding for NKCC2 result in Bartter's syndrome, which is characterized by hypokalemic alkalosis, hyponatremia and hypotension (Simon et al., 1996).

The less studied members of the KCC family (KCC1, KCC3, and KCC4) are also expressed in the developing brain. KCC1 mRNA was exclusively detected in the choroid plexus during mouse brain development (Li et al., 2002), but mRNA levels have been found in neuronal and glial cells in diverse regions (olfactory bulb, hippocampus, choroid plexus, posterior hypothalamic nucleus) of the adult rat CNS in vivo (Kanaka et al., 2001). Interestingly, KCC1 negatively regulates NGF-induced neurite outgrowth in vitro in PC12 cells (Nagao et al., 2012). KCC3 mRNA is weakly represented in the embryonic rodent brain (Li et al., 2002), but both the mRNA and the protein are widely present in adult cortical, hippocampal, brainstem and cerebellar Purkinje neurons (Pearson et al., 2001; Shekarabi et al., 2011). Moreover, KCC3 protein is expressed in white matter-rich structures in the rodent brain, spinal cord and peripheral nerves, indicating a role of KCC3 in myelination (Pearson et al., 2001).

Furthermore, KCC3 regulates the cell volume in mouse peripheral nerve fibers (Flores et al., 2018). In agreement with the previous findings, KCC3$^{-/-}$ mice exhibited axonal swelling, hypomyelination, and demyelination in sciatic nerves (Byun and Delpire, 2007; Howard et al., 2002). Interestingly, these mice recapitulate most of the symptoms of human peripheral neuropathy associated with agenesis of the corpus callosum (ACCPN, also known as Andermann syndrome). This severe sensorimotor neuropathy is characterized by locomotor abnormalities and areflexia and has been associated with loss-of-function mutations in KCC3 gene (Bowerman et al., 2017; Howard et al., 2002; Uyanik et al., 2006).

KCC4 is highly expressed in the embryonic mouse brain, including choroid plexus, peripheral ganglia, ventricular zones and the nucleus of the trigeminal nerve (Li et al., 2002). Along with NKCC1, KCC4 plays a role in cochlear development, as KCC4 KO mice exhibit deafness. KCC4 loss possibly exerts that effect by causing the death of hair cells by osmotic perturbation or membrane depolarization (Boettger et al., 2002).

Spatiotemporal expression of the KCC transporters has been studied also in humans. In particular, KCC1 mRNA was first observed in the cortex of the cerebellum at embryonic stages (between PCW 10–13) and also found in other brain regions (hippocampus, striatum, and thalamus between PCW 21 and birth; Sedmak et al., 2016). KCC3 mRNA was described in the cortex, cerebellum, hippocampus, amygdala, striatum, and thalamus in all the stages of development and adulthood (age range: 5 PCW–82 years; Sedmak et al., 2016). KCC4 mRNA was detected at low levels in the cortex both at prenatal and postnatal ages (Kaila et al., 2014), but not detected in a subsequent study in any brain region (Sedmak et al., 2016). NKCC2 mRNA was not detected in any brain region (Sedmak et al., 2016).

Chloride transporters in neurodevelopmental disorders

Neurodevelopmental disorders (NDs) are chronic psychiatric/neurological conditions that affect 4–5% of the population (Mitchell, 2011). In general, NDs result from an altered developmental processes induced by both genetic and environmental factors, which cause defective growth of the central nervous system. Although they display very different etiologies, most of the NDs share a number of features (e.g., impairments in learning, memory, emotional regulation, sociality, and self-control) and some comorbidity (e.g., increased seizure susceptibility and sleep disorders). Interestingly, many of these common features have been associated with common alterations in GABAergic transmission. In particular, several studies demonstrated a high intracellular chloride concentration and a depolarizing GABA action

attributable to an altered NKCC1/KCC2 ratio in a wide range of NDs, including epilepsy, Dravet syndrome, autism spectrum disorders, Asperger syndrome, Rett syndrome, Fragile X syndrome, schizophrenia, tuberous sclerosis complex, traumatic brain injury, and Down syndrome (Ben-Ari, 2017; Jaggi et al., 2015; Medina et al., 2014; Schulte et al., 2018; Wu et al., 2016a).

Interestingly, restoration of physiological Cl^- concentration by pharmacological intervention aimed at inhibiting NKCC1 or enhancing KCC2 activity has led to positive outcomes in rodent models and patients with these conditions (Ben-Ari, 2017; Jaggi et al., 2015; Medina et al., 2014; Schulte et al., 2018). Currently, the most used approach to restore low intracellular Cl^- concentration in mouse models of brain pathologies as well as in clinical studies in patients has been the inhibition of NKCC1 with bumetanide. Another recently investigated option to restore intracellular Cl^- concentration has been the enhancement of KCC2 activity by the compound CLP257. This has been explored in cultured cell lines and in spinal cord slices obtained from rats with peripheral nerve injury, a condition characterized by KCC2 hypofunction (Gagnon et al., 2013). Interestingly, CLP257, by modulating KCC2 activity, was also able to exert an antinociceptive action in rats with peripheral nerve injury (Gagnon et al., 2013). Nevertheless, a recent study showed that CLP257 was not able to modulate KCC2 activity in vitro in the same cultured cell line used previously, opening the possibility that the behavioral effects of CLP257 observed by Gagnon and colleagues may be independent of KCC2 modulation (Cardarelli et al., 2017). Again, this study was challenged by Gagnon and colleagues, who replied to the Cardarelli and co-workers objection confirming their previous findings (Gagnon et al., 2017), thus indicating the need for further investigation.

Finally, recent studies evaluated other components involved in NKCC1 and KCC2 regulation that could be considered in the future as possible therapeutic targets. For example, the insulin-like growth factor-1 was able to decrease the NKCC1/KCC2 ratio in developing rat neurons in vivo, promoting the GABA switch from depolarizing to hyperpolarizing (Baroncelli et al., 2017). Moreover, the kinase WNK-SPAK, which can activate NKCC1 and deactivate KCC2 through its phosphorylation state (Kahle et al., 2010), could also be an interesting target to modulate the NKCC1/KCC2 ratio (de Los Heros et al., 2014; Kahle et al., 2015).

Here, we give a brief description of the involvement of altered NKCC1 and KCC2 expression/function in the pathogenesis of some neurodevelopmental disorders, considering some examples of therapeutic approaches. We describe more in detail the case of the Down syndrome at the end of this section.

Epilepsy

Epilepsy is a neurological disorder characterized by epileptic seizures. These are caused by altered, excessive or hypersynchronous neuronal activity in the brain (Chang and Lowenstein, 2003). Several pieces of evidence suggest that neuronal hyperexcitability and hypersynchronization is the result of an alteration of the delicate balance between excitatory and inhibitory synaptic activity. Interestingly, an imbalance in NKCC1 and KCC2 activity together with depolarizing GABAergic action have been observed in several animal models of epilepsy (Ben-Ari, 2017; Di Cristo et al., 2018). The first lines of evidence about the involvement of CCCs in epileptogenic activity came from the late 90s, when four independent studies found that the antagonization of NKCC1 with furosemide or bumetanide caused a block of epileptic activity both in vitro and in vivo in rats (Hochman et al., 1995, 1999; Hochman and Schwartzkroin, 2000; Schwartzkroin et al., 1998). In 2002, four other works demonstrated the direct relationship between NKCC1, KCC2 and epileptic pathogenesis. In particular, high NKCC1 expression was indicated as a factor influencing the increased susceptibility to seizures in the developing brain. In this study, bumetanide administration was able to rescue epileptiform activity both in vitro and in vivo during development in rodents (Dzhala et al., 2005). Moreover, increased expression of NKCC1 was found in the amygdala-kindling model of seizures in rats (Okabe et al., 2002). Furthermore, a decrease in KCC2 expression was found in the mouse hippocampus after kindling-induced seizures (Rivera et al., 2002). Finally, mice deficient in KCC2 showed frequent seizures (Woo et al., 2002). This may be possibly due to a shift in the E_{Cl}, which leads to impaired efficacy of $GABA_AR$-mediated inhibition and/or to less reuptake of potassium and chloride during high-frequency spikes (Woo et al., 2002). Stemming from these first works, several other studies in rodent models confirmed the involvement of an altered NKCC1/KCC2 ratio in the pathogenesis of epilepsy. A number of these studies also confirmed positive outcomes upon bumetanide treatment (Almeida et al., 2011; Amadeo et al., 2018; Baek et al., 2016; Cleary et al., 2013; Dzhala et al., 2008, 2010; Edwards et al., 2010; Eftekhari et al., 2014a; Hu et al., 2017; Kelley et al., 2018; Koyama et al., 2012; Li et al., 2008; Loscher et al., 2013; MacKenzie and Maguire, 2015; MacKenzie et al., 2016; Marguet et al., 2015; Mazarati et al., 2009; Nardou et al., 2009; Reid et al., 2013; Robel et al., 2015; Santos et al., 2017; Sivakumaran and Maguire, 2016; Tao et al., 2016; Tollner et al., 2015a; Wang et al., 2017; Zhang et al., 2016a).

Interestingly, an imbalance in the NKCC1/KCC2 ratio is also present in human patients. First, upregulation of NKCC1 and/or downregulation of KCC2 was found in the hippocampal subiculum and hippocampi obtained from patients affected by temporal lobe epilepsy (Huberfeld et al., 2015, 2007; Munoz et al., 2007; Palma et al., 2006; Sen et al., 2007). Then, other studies found altered

expression of NKCC1 and/or KCC2 in the cortical malformation of patients affected by medically intractable epilepsy (Aronica et al., 2007; Sen et al., 2007; Shimizu-Okabe et al., 2011) in hypothalamic hamartoma, a rare epileptogenic lesion associated with gelastic seizures (Kim et al., 2008), in cortical samples from epileptic children (Jansen et al., 2010), and in peritumoral tissues with high seizure susceptibility (Conti et al., 2011; Pallud et al., 2014). Notably, an increased expression of NKCC1 and a decreased expression of KCC2 were observed also in brain samples obtained from patients affected by Dravet syndrome, an infantile encephalopathy characterized by severe epilepsy and cognitive impairment (Ruffolo et al., 2018). Interestingly, bumetanide treatment ameliorated seizure frequency in temporal lobe epilepsy (Eftekhari et al., 2014a). Moreover, bumetanide was able to reduce seizure duration and frequency in a child affected by intractable multifocal seizures (Kahle et al., 2009). Nevertheless, the NEMO trial, assessing the efficacy and safety of the use of bumetanide for the treatment of acute neonatal encephalopathy seizures (Pressler et al., 2015), was recently interrupted due to poor bumetanide antiepileptic action and ototoxicity (Ben-Ari et al., 2016). Moreover, the involvement of KCC2 in the pathogenesis of epilepsy has been recently questioned based on conflicting results showing increased KCC2 expression in epileptic brain tissue from both human (Jansen et al., 2010; Karlocai et al., 2016) and rodent models (Awad et al., 2016; Galanopoulou, 2008; Khirug et al., 2010). Nevertheless, the conflicting results showed both decreased and increased KCC2 expression in epilepsy; this discrepancy could depend on brain region, stage of disease, gender, or the influence of seizures themselves (Di Cristo et al., 2018). Indeed, KCC2 activity causes potassium elevation in the extracellular compartment, which could contribute to the lowering of the threshold for the generation of the seizures and to the synchronization of the epileptiform discharges (Di Cristo et al., 2018). Thus, although the involvement of alterations of NKCC1 and KCC2 expression/activation in epilepsy is clearly demonstrated, deeper studies to better investigate their delicate modulation and assess the possibility of targeting them with pharmacological approaches are still required.

Autism spectrum disorders

Autism spectrum disorders (ASD) are a group of syndromes characterized by different etiologies, but common core symptoms (e.g., repetitive behaviors, deficits in social interaction and language impairment; Pizzarelli and Cherubini, 2011), suggesting that possibly there are common mechanisms underlying ASD pathology. Moreover, ASD can be comorbid with other neurodevelopmental syndromes such as epilepsy (Lewis et al., 2018), Rett syndrome (Percy, 2011), Fragile X syndrome (Kaufmann et al., 2017), or Down syndrome (Moss et al., 2013). Several pieces of evidence, both from rodent models and

humans, indicate that commonly altered GABAergic transmission could underlie ASD pathology (Cellot and Cherubini, 2014). In particular, the pioneering observation of a paradoxical effect upon the administration of $GABA_A$-signaling-enhancing benzodiazepine diazepam in autistic children (e.g., anxiety and aggression; Marrosu et al., 1987), suggested the possibility of depolarizing GABA action in ASD. This idea prompted researchers to test whether the inhibition of NKCC1 by bumetanide could be a valid therapeutic strategy in five autistic children (Lemonnier and Ben-Ari, 2010). The amelioration of some behavioral aspect related to ASD upon bumetanide treatment opened the way for a larger clinical trial designed for 54 autistic patients (Lemonnier et al., 2012) and consequently a phase II clinical study (Lemonnier et al., 2017). These studies confirmed that bumetanide is able to ameliorate the core symptoms of ASD measured by the Childhood Autism Rating Scale (CARS). Moreover, bumetanide resulted efficient in the treatment of a young girl with Asperger syndrome, a neurodevelopmental disorder belonging to ASD (Grandgeorge et al., 2014). In parallel to the clinical studies, KCC2 expression was downregulated in the VPA rat model of ASD (Eftekhari et al., 2014b; Tyzio et al., 2014). Interestingly, bumetanide administration in VPA-treated pregnant rats resulted in the rescue of core behaviors related to ASD in their offspring (Eftekhari et al., 2014b; Tyzio et al., 2014). Nevertheless, the lack of preclinical studies addressing bumetanide treatment at the developmental stages comparable to those of patients enrolled in clinical trials and the paucity of mouse models of autism tested among the many that exist, highlights the need for further investigation.

Rett syndrome

Rett syndrome (RTT) is a neurodevelopmental disorder caused by mutations in the X-linked Methyl-CpG-binding protein (MECP2) gene. Mecp2 binds to methylated DNA and regulates the transcription of a large number of genes. Individuals affected by RTT grow normally until the age of 6–18 months but then develop various symptoms (e.g., cognitive impairment, seizures, altered motor function and stereotype behaviors; Ehinger et al., 2018). As for autism, several pieces of evidence indicate a possible alteration in the GABAergic signaling in RTT rodent models and humans (Cellot and Cherubini, 2014). The first evidence regarding alterations in the NKCC1/KCC2 balance came from a study of corticospinal fluid (CSF) obtained from RTT patients, where a decreased level of KCC2 expression was found (Duarte et al., 2013). More recently, deficits in KCC2 expression have been found in human RTT patient stem cell-derived neurons (Tang et al., 2016) and in a mouse model of RTT (Banerjee et al., 2016). Interestingly, insulin-like growth factor-1 (IGF1) treatment ameliorated the severity of the syndrome both in RTT mouse models (Castro et al., 2014; Tropea et al., 2009) and in RTT patients (Khwaja et al., 2014; Pini et al., 2016), thus suggesting

its implementation in the treatment of NDs (Bou Khalil, 2017). Nevertheless, further investigation of the NKCC1/KCC2 ratio and its consequence on GABA signaling are needed to better clarify the role of the two CCCs in the pathogenesis of RTT and their possible involvement as therapeutic targets.

Fragile X syndrome

Fragile X syndrome (FXS) is a genetic disorder caused by mutations in the X-linked FMR1 gene encoding for Fragile X mental retardation protein (FMRP). FMRP is a regulator of the translation of several mRNAs. FXS individuals show cognitive deficits, autistic behavior, hypersensitivity to sensory stimuli and comorbidity with epilepsy (Morel et al., 2018). These symptoms led researchers to hypothesize an excitatory/inhibitory imbalance, as previously observed in ASD and epilepsy. In particular, driven by the positive outcome of the earlier pilot study on autistic patients (Lemonnier et al., 2012), the same authors treated a FXS child with bumetanide (Lemonnier et al., 2013). Interestingly, bumetanide administration resulted in the amelioration of the score of each of the 5 clinical tests performed to probe autistic core symptoms (Lemonnier et al., 2013), opening the route to larger clinical trials. In agreement with the clinical study, FXS mice showed a delay in the developmental switch of GABA polarity from depolarizing to hyperpolarizing, due to increased expression of NKCC1 (He et al., 2014). The same year, Tyzio and colleagues found increased Cl^- concentrations in hippocampal slices from FXS mice at P15 and P30 due to a decreased level of KCC2. Fetal treatment with bumetanide right before birth was able to recover the intracellular Cl^- concentration, GABAergic transmission and the behavioral features related to autism later in life (Eftekhari et al., 2014b; Tyzio et al., 2014). Recently, treatment of FXS mice with bumetanide during the critical period of somatosensory cortex plasticity rectified GABA polarity and synaptic plasticity and allowed long-lasting restoration of proper somatosensory-circuit formation (He et al., 2018). Moreover, a recent study found that bumetanide treatment by itself was insufficient to completely rescue social impairment in the automated tube test in FXS mice, suggesting the need for a combination therapy (Zeidler et al., 2017). Nevertheless, in the same study, the combination of the genetic reduction of mGluR5 expression together with bumetanide treatment worsened social impairment, indicating that the combination therapy needs to be better investigated in terms of drug type, targeting pathway and time window of administration (Zeidler et al., 2017). Although there are only a few studies in animal models and in humans, the abovementioned evidence confirms the involvement of the NKCC1/KCC2 imbalance in the pathogenesis of FXS syndrome and their modulation as a possible therapeutic strategy.

Schizophrenia

Schizophrenia is a neurodevelopmental disorder characterized by psychosis and cognitive impairments, leading to disability and premature mortality (Lewis, 2012). In particular, the clinical manifestations can be divided into three categories: positive symptoms (e.g., hallucinations), negative symptoms (e.g., depression and apathy), and cognitive symptoms (Lewis, 2012). The etiology of schizophrenia is still under investigation, but a large body of literature agrees on the contribution of both genetic and environmental factors. GABAergic transmission seems again to play an important role in the pathogenesis of this ND (Balu and Coyle, 2011). In particular, the first pieces of evidence of impaired Cl^- homeostasis in schizophrenia came from a study on the prefrontal cortex (and later the hippocampus) of schizophrenic patients, where NKCC1 expression was increased (Dean et al., 2007; Hyde et al., 2011). A few years later, alterations in SLC12A2 and SLC12A5 genes, encoding for NKCC1 and KCC2, respectively, were indicated as susceptibility genes for schizophrenia development in patients (Kim et al., 2012; Merner et al., 2015, 2016; Potkin et al., 2009). Furthermore, increased expression of two kinases regulating NKCC1 and KCC2 activity, OXSR1 and WNK3, was found in the prefrontal cortex of schizophrenic subjects, indicating a possible increase in NKCC1 activity and a decrease in KCC2 function in schizophrenic patients (Arion and Lewis, 2011). In addition, an altered NKCC1/KCC2 ratio was described in two different mouse models of schizophrenia (Larimore et al., 2017; Yang et al., 2015). Finally, an interplay between NKCC1 and the protein Disrupted in schizophrenia 1 (DISC1, an intrinsic regulator of neurogenesis implicated in schizophrenia) has been demonstrated to be fundamental for the regulation of the dendritic development of newborn neurons during adult neurogenesis in the mouse hippocampus (Kim et al., 2012), suggesting possible involvement of NKCC1 in the pathogenic mechanisms underlying schizophrenia.

Of note, in vitro evidence from Amin and coworkers revealed an imbalance in NKCC1 and KCC2 expression also in DiGeorge Syndrome (a condition conferring high risk of schizophrenia), which caused hyperexcitability of the network recovered by bumetanide application to the neuronal culture (Amin et al., 2017).

Bumetanide treatment in schizophrenic patients reduced the severity of the symptoms and hallucinations (Lemonnier et al., 2016; Rahmanzadeh et al., 2017a), without ameliorating the total score of the general positive and negative syndrome scale (PANSS) and the brief psychiatric rating scale (BPRS) (Rahmanzadeh et al., 2017b). Interestingly, intranasal administration of oxytocin reduced the severity of symptoms in schizophrenic patients in several studies (Brambilla et al., 2016; Davis et al., 2014, 2013; Feifel et al., 2012, 2010;

Fischer-Shofty et al., 2013; Gibson et al., 2014; Goldman et al., 2011; Lee et al., 2013; Modabbernia et al., 2013; Ota et al., 2018; Pedersen et al., 2011; Shin et al., 2015; Woolley et al., 2017, 2014), but see (Cacciotti-Saija et al., 2015; Caravaggio et al., 2017; Dagani et al., 2016; Horta de Macedo et al., 2014; Jarskog et al., 2017). In light of the ability of oxytocin to regulate GABA signaling in fetal and newborn rodents (Ben-Ari, 2018; Eftekhari et al., 2014b; Khazipov et al., 2008; Leonzino et al., 2016; Tyzio et al., 2006, 2014), it is tempting to hypothesize that oxytocin exerts its therapeutic effect on schizophrenic patients also by regulating CCCs. A deeper investigation of the molecular mechanisms underlying the possible relation between the oxytocin system and CCCs could open new avenues for the treatment of schizophrenia and other NDs.

Tuberous sclerosis complex

Tuberous sclerosis complex (TSC) is a multiorgan genetic disorder caused by loss of function mutations of the TSC1 or TSC2 genes (van Slegtenhorst et al., 1997). This pathology is characterized by the presence of cortical tubers (i.e., dysplastic lesions), source of focal epilepsy, autistic behaviors and intellectual disability. Given the imbalance of the NKCC1/KCC2 ratio in epilepsy (Schulte et al., 2018), the investigation of CCCs in tuberous sclerosis has gained interest. TSC patients present an increased NKCC1/KCC2 ratio in extracts from cortical tubers (Ruffolo et al., 2016; Talos et al., 2012). An altered GABA reversal potential was also described in Xenopus oocytes injected with membranes from TSC patient cortical tissues (Ruffolo et al., 2016). Altogether, these studies suggest a possible involvement of NKCC1/KCC2 imbalance in the pathogenesis of TSC in patients. Nevertheless, a better investigation of both the pathogenic mechanisms and possible therapies needs to be performed in rodent models.

Neurodevelopmental abnormalities caused by traumatic brain injury

Traumatic brain injury (TBI) is caused by an injury to the brain due to external objects or forces. When TBI occurs in early childhood, the cortical and subcortical lesions lead to altered neurodevelopmental processes and consequent cognitive defects persisting for the lifetime of the individual (Bonnier et al., 2007; Jonsson et al., 2013; Keenan et al., 2007). The neurodevelopmental damages occurring after a TBI are the result of a cascade of events, called secondary brain injury, including damage of the blood-brain barrier, inflammation, excitotoxicity, edema, ischemia, and neuronal damage (e.g., excitotoxicity, aberrant ionic homeostasis, axonal disconnection, and death; Ghajar, 2000; Park et al., 2008). One of the mechanisms underlying this cascade of events is possibly an imbalance of NKCC1 and KCC2 expression and function. Indeed, three independent studies found that NKCC1 was upregulated in the hippocampus and choroid

plexus of traumatic brain injury rat models and that bumetanide administration decreased the inflammatory response and neuronal damage (Lu et al., 2008, 2006, 2007). Then, other studies confirmed the fundamental role of NKCC1 in TBI-induced rodent hippocampal aberrant neurogenesis (Lu et al., 2015), neuronal and astrocytic apoptosis (Hui et al., 2016; Zhang et al., 2017b), cerebral edema (Lu et al., 2017; Zhang et al., 2016b), seizures (Liang and Huang, 2017; Wang et al., 2017), BBB disruption (Zhang et al., 2017a), and microvascular failure (Simard et al., 2010). Finally, a recent work described decreased KCC2 expression in the rat parietal cortex after TBI, which was rescued by melatonin administration leading to amelioration of neural apoptosis and brain edema (Wu et al., 2016b). The deep understanding of the involvement of both NKCC1 and KCC2 in the secondary brain injury upon TBI suggests timely pharmacological interventions to prevent the consequent neurodevelopmental alterations observed in TBI children.

Chloride transporters in Down syndrome

Down syndrome (DS) is caused by the triplication of human chromosome 21 (Hsa21) and it is one of the most common genetic causes of intellectual disability and congenital birth defects. Many health issues characterize persons with DS (Antonarakis and Epstein, 2006; Desai, 1997; Nadel, 2003; Parker et al., 2010), with almost all individuals presenting with cognitive deficits (Dierssen, 2012; Edgin et al., 2012; Pennington et al., 2003; Vicari et al., 2013).

Several studies investigated possible mechanisms involved in cognitive impairment, taking advantage of diverse murine genetic models of DS (Dierssen, 2012). The Ts65Dn mouse (Reeves et al., 1995) is the most characterized and widely used. These mice are characterized by the presence of an extra chromosome derived from mouse chromosome 16, representing the long arm of human chromosome 21, fused to the centromere of the murine chromosome 17 (Antonarakis et al., 2004). Interestingly, Ts65Dn mice recapitulate many features of DS. In particular, these mice show impairment in neuronal development (Belichenko et al., 2004; Chakrabarti et al., 2010, 2007; Contestabile et al., 2010, 2007), defects of synaptic plasticity (Contestabile et al., 2013; Costa and Grybko, 2005; Kleschevnikov et al., 2004; Siarey et al., 1999, 1997), impaired hippocampus-dependent memory functions (Contestabile et al., 2013; Costa et al., 2008; Fernandez et al., 2007; Reeves et al., 1995), hyperactivity (Escorihuela et al., 1995; Reeves et al., 1995; Sago et al., 2000), and sleep disorders (Colas et al., 2008; Das et al., 2015; Stewart et al., 2007).

Down syndrome and GABAergic transmission

The first lines of evidence regarding the involvement of defective GABAergic transmission in DS found an increased number of GABAergic interneurons in the cortex and hippocampus of adult and adolescent Ts65Dn mice, respectively (Chakrabarti et al., 2010; Perez-Cremades et al., 2010). These alterations were accompanied by an increase in spontaneous GABAergic postsynaptic events in CA1 pyramidal neurons of adult Ts65Dn mice (Chakrabarti et al., 2010). Interestingly, subsequent provided seemingly contrasting and often inconsistent results, depending on the parameters analyzed, hippocampal subregion and age of the animals. For instance, electrophysiological experiments did not find any alterations nor in the frequency of miniature inhibitory postsynaptic currents (mIPSC), neither in the release probability at GABAergic synapses. Moreover evoked $GABA_A$ transmission in the hippocampal CA1 region of adult Ts65Dn mice (Best et al., 2012; Chakrabarti et al., 2010), as well as electron microscopy and immunohistochemical studies on hippocampus of adult Ts65Dn mice did not reveal any alteration in the density GABAergic terminals and synapses, but only on their distribution (Belichenko et al., 2009b, 2004; Kleschevnikov et al., 2012b; Kurt et al., 2000, 2004). Conversely, mIPSC frequency was found increased in the CA1 region of Ts65Dn pups (Mitra et al., 2012) and in DG of Ts65Dn adults (Kleschevnikov et al., 2012b, 2004), whereas it was found decreased in hippocampal CA3 region of Ts65Dn mice (Hanson et al., 2007; Stagni et al., 2013) and GABAergic synaptic density was found increased in the hippocampal DG of adult Ts65Dn mice (Garcia-Cerro et al., 2014; Martinez-Cue et al., 2013; Mojabi et al., 2016). Interestingly, studies of DS autoptic brain samples and analysis of cortical neuronal progenitors obtained from DS individuals have shown a general reduction in the GABAergic system at various levels (i.e., defects in interneuron neurogenesis, reduced number and size in cortical calbindin and parvalbumin neurons) (Bhattacharyya et al., 2009; Kobayashi et al., 1990; Ross et al., 1984), in seemingly inconsistence with the first evidence describing increased GABAergic transmission in Ts65Dn mice.

Thus, the evidence in TS65Dn mice and brain samples taken altogether suggests that besides an increased number of GABAergic interneurons, hippocampal subregion and age-dependent differences, together with compensatory mechanisms and a general increase in the excitability of the interneurons may be present in DS (Contestabile et al., 2017).

Interestingly, further studies demonstrated that the altered GABAergic transmission affected synaptic plasticity in DS mice, experimentally measured with the long-term potentiation (LTP) protocols in acute brain slices of adult Ts65Dn mice (Belichenko et al., 2009a, 2015, 2007; Costa and Grybko, 2005; Fernandez et al., 2007; Kleschevnikov et al., 2012a, 2004).

Given that several pieces of evidence suggested that the cognitive deficits and abnormalities in synaptic plasticity observed in Ts65Dn mice derive, at least in part, from an excess of $GABA_A$-mediated neurotransmission in the hippocampal circuitry (i.e., spontaneous GABAergic postsynaptic events in CA1, mIPSC frequency and evoked GABAergic transmission in DG), diverse studies evaluated $GABA_A$ receptors as a possible therapeutic target to rescue cognitive impairment in DS. For 10 years, numerous independent groups tested $GABA_A$ receptor inhibitors targeting diverse subunits and consistently found a rescue in LTP and hippocampal cognitive abilities in Ts65Dn mice (Braudeau et al., 2011a,b; Fernandez et al., 2007; Martinez-Cue et al., 2014, 2013; Mohler, 2012; Potier et al., 2014; Rueda et al., 2008). Similar results were obtained by treating Ts65Dn animals with fluoxetine, an inhibitor of serotonin reuptake (Begenisic et al., 2014; Bianchi et al., 2010; Guidi et al., 2014; Stagni et al., 2015), but see (Heinen et al., 2012) or exposing the Ts65Dn animals to an enriched environment (Begenisic et al., 2015, 2011; Martinez-Cue et al., 2002, 2005). Since both fluoxetine and exposure to an enriched environment reduced GABAergic signaling (Baroncelli et al., 2010; Begenisic et al., 2014, 2015, 2011; Caiati and Cherubini, 2013; Maya Vetencourt et al., 2008; Mendez et al., 2012; Sale et al., 2007), it is possible that the effects on Ts65Dn mice may be due, at least in part, to modulation of GABAergic transmission, as in all the studies reported above for $GABA_A$ receptor inhibitors.

These lines of evidence reinforce the hypothesis of a causal link between the increased GABAergic transmission, synaptic plasticity abnormalities and cognitive deficits of DS mice (Chakrabarti et al., 2010; Kleschevnikov et al., 2004) and highlight GABAergic transmission as a possible therapeutic target in DS. On the other hand, both individuals with DS and DS mice show an increased susceptibility to seizures (Arya et al., 2011; Gholipour et al., 2017; Goldberg-Stern et al., 2001; Lott, 2012; Lott and Dierssen, 2010; Rissman and Mobley, 2011; Robertson et al., 2015; Smigielska-Kuzia et al., 2009; Stafstrom et al., 1991; Westmark et al., 2010) and hyperactivity (Deidda et al., 2015b; Escorihuela et al., 1995; Moss, 2017; Pueschel et al., 1991; Reeves et al., 1995; Sago et al., 2000), and individuals with DS often show anxiety traits (Dekker et al., 2018; Haddad et al., 2018; Vicari et al., 2013), pointing to excess excitation rather than inhibition in DS.

NKCC1 is implicated in depolarizing $GABA_AR$ signaling in Down syndrome

In 2015, Deidda and colleagues proposed a new perspective about $GABA_AR$ transmission in DS (Deidda et al., 2015b). In their work, the efficacy and polarity of $GABA_AR$ signaling were investigated in adult Ts65Dn mice. Surprisingly, they found that GABAergic transmission was depolarizing and mostly

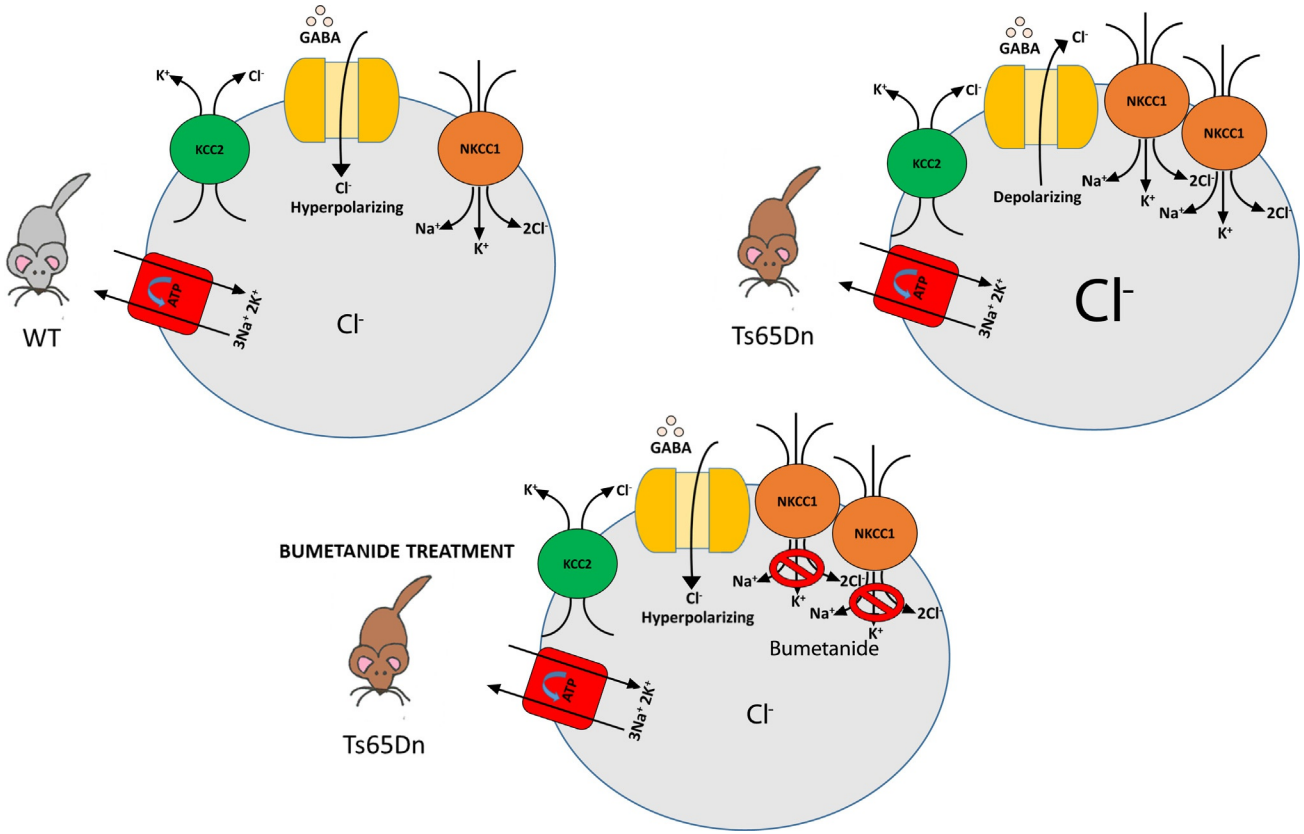
excitatory rather than hyperpolarizing and inhibitory in adult DS mice. In particular, they described an increase in spike frequency in Ts65Dn hippocampal and neocortex acute slices in comparison to WT, both in baseline conditions and upon application of GABA. Accordingly, blockade of endogenous $GABA_A$ signaling by the application of the $GABA_AR$ antagonist bicuculline resulted in a reduction in the spike frequency in neurons from Ts65Dn brain slices.

The excitatory action of GABA in Ts65Dn brain slices was accompanied by a shift in the reversal potential for $GABA_AR$-driven Cl^- currents (E_{Cl}) to more positive potentials. Indeed, taking advantage of the gramicidin-perforated patch clamp, a technique that allows maintenance of the endogenous intracellular Cl^- concentration, they observed that Ts65Dn neurons exhibited a less negative E_{Cl} (−58 mV) in comparison to WT neurons (−66 mV). The use of gramicidin-perforated patch-clamp was key, considering that another study conducted to investigate E_{Cl} in Ts65Dn mice by whole-cell patch-clamp did not detect these differences possibly due to alterations of intracellular chloride concentration caused by dilution from the pipette solution (Kleschevnikov et al., 2012b). Of note, an E_{Cl} value above the membrane resting potential suggests an outward Cl^- current. This was indeed described by Deidda and colleagues by Cl^- imaging in CA1 neurons from Ts65Dn acute brain slice.

Interestingly, the same study found that the defective GABAergic signaling was due to an increased expression of NKCC1 protein, which they found in the entire hippocampus, the CA3-CA1 subregion and cortices of adult Ts65Dn mice compared to WT littermates. Interestingly, Deidda and coworkers found increased NKCC1 expression also in hippocampi from DS individuals, providing a parallel between the animal model and humans. Conversely, no changes in KCC2 protein expression both in Ts65Dn mice and DS individuals were detected. Notably, the authors did not find a significant increase in NKCC1 mRNA in adult Ts65Dn mice.

Bumetanide treatment rescues the altered GABAergic transmission, synaptic plasticity and cognitive deficits in Ts65Dn mice

Considering that the increased expression of NKCC1 is the possible cause of the aberrant GABAergic transmission in Ts65Dn mice, Deidda and colleagues evaluated NKCC1 inhibition by bumetanide as a potential therapeutic strategy. Bath application of bumetanide was able to rescue E_{Cl}, with a reduction of spontaneous spiking activity and a decrease in the GABA-induced spike frequency in acute hippocampal slices of adult Ts65Dn mice. Conversely, there was no significant effect of bumetanide application in WT mice, confirming that the shift of E_{Cl} was responsible for depolarizing $GABA_AR$ signaling in adult Ts65Dn mice.


GABA
K+
Cl-
KCC2
NKCC1
Cl-
Hyperpolarizing
Na+
K+
2Cl-
ATP
3Na+ 2K+
Cl-
WT
Ts65Dn
Depolarizing
BUMETANIDE TREATMENT
Bumetanide

Moreover, bumetanide bath application to acute brain slices was able to recover the hippocampal CA1-CA3 LTP to WT levels, with no effect on the LTP in WT mice. Finally, Deidda and colleagues tested Ts65Dn mice and their WT littermates in three independent behavioral tasks to assess hippocampus-dependent long-term explicit memory after either an acute (1 time only), subchronic (1 week) or a chronic (4 weeks) systemic (intraperitoneal) treatment with bumetanide. Interestingly, they proved that all three treatments with bumetanide were able to fully recover the poor associative memory of Ts65Dn mice in the contextual fear conditioning test. Moreover, bumetanide was able to rescue the performance of Ts65Dn mice to the level of WT mice in the object-location test, showing a full recovery of spatial-memory performance. Finally, bumetanide administration was also able to rescue the novel-discrimination memory of Ts65Dn mice in the novel object recognition test (Deidda et al., 2015b).

Notably, bumetanide exerted acute activity on NKCC1 and it did not provide long-lasting effects. Indeed, a drug withdrawal experimental protocol (i.e., a week of bumetanide washout after a four-week treatment) completely abolished the rescue observed in both LTP and behavioral tasks, indicating the requirement for chronic treatment (Deidda et al., 2015b).

Concluding remarks

From Deidda and colleagues' work to the other several studies, we have highlighted here a clear implication for the disrupted NKCC1/KCC2 expression ratio in the pathogenesis of neurodevelopmental disorders such as Down syndrome, epilepsy, ASD, Rett syndrome, Fragile X, schizophrenia, tuberous sclerosis complex, and neurodevelopmental abnormalities caused by traumatic brain injury. Accordingly, the inhibition of NKCC1 by the FDA-approved diuretic bumetanide is able to recover pathological phenotypes associated with the aforementioned neurodevelopmental conditions in rodent models and/or human subjects, as highlighted above. This highlights the tremendous potential for repurposing (Strittmatter, 2014) of bumetanide in the treatment of a number of neurodevelopmental disorders. Nevertheless, there are still some open issues that need to be addressed.

First, the mechanisms underlying the dysregulation of the NKCC1/KCC2 expression ratio in pathology, as well as its physiological variation during early development are still mostly unknown and insufficiently investigated (Schulte et al., 2018). Interestingly, the discrepancy between the level of NKCC1 mRNA (not increased) and the protein (increased) observed in brain homogenates from DS mice suggests possible posttranscriptional regulatory mechanisms (Deidda et al., 2015b). This could open new avenues for the investigation of the mechanism regulating NKCC1 expression in pathological conditions and

the possibility of new therapeutic targets for Down syndrome and the other ND characterized by an aberrant NKCC1/KCC2 expression ratio.

Second, bumetanide is a strong diuretic, which is currently only used in hospitals to mainly treat the consequences of heart failure. Thus, the use of a strong diuretic to treat patients with severe behavioral impairments as in ND may seriously jeopardize drug compliance during chronic treatment. Bumetanide exerts its diuretic actions by blocking NKCC2, which leads to ionic imbalance. Moreover, NKCC2 is also expressed in vasopressinergic and oxytocinergic neurons in the hypothalamo-neurohypophyseal system and in the vestibular system, increasing the potential side effects of chronic bumetanide treatment. Hence, a new drug selective for NKCC1 and devoid of unfavorable diuresis, electrolyte imbalance, and unwanted effects on the hypothalamo-neurohypophyseal system and auditory system is required to ameliorate the symptoms of the subjects affected by ND. In particular, the effects on the auditory system have proved critical for the treatment of infants. Indeed, one clinical trial for the repurposing of bumetanide for the treatment of acute neonatal encephalopathy seizures was suspended due to induced deafness in some treated subjects (Ben-Ari et al., 2016).

Third, a number of studies highlighted bumetanide's poor blood-brain barrier penetration (Brandt et al., 2010; Cleary et al., 2013; Puskarjov et al., 2014a; Tollner et al., 2015b; Topfer et al., 2014) and recent investigations considered bumetanide's low levels in the brain after systemic administration as incompatible with NKCC1 inhibition, thus questioning the brain expressed-NKCC1 as the target of bumetanide in ND (Romermann et al., 2017; Wang et al., 2015). Further studies are required to confirm NKCC1 as a therapeutic target in the brain and to elucidate the mechanisms of action of bumetanide in brain-related behaviors.

Finally, most of the brain defects occurring in DS as well as in the other ND described above originate during development. Thus, the deep investigation of NKCC1 and KCC2 expression and function in neural maturation and circuit formation could elucidate the mechanisms underlying the pathogenesis of these conditions. Moreover, this could open the possibility to search for more efficient, timely, and early therapeutic strategies. Indeed, acting when neuronal networks are still plastic during development could ameliorate symptoms that treatments in adult ages were not able to reverse, possibly due to preexisting miswiring in the neuronal circuit. For example, bumetanide treatment in adult Ts65Dn mice failed to rescue hyperactivity and seizure susceptibility, whereas an earlier treatment could be beneficial (Deidda et al., 2015b). Moreover, early pharmacological treatments in NDs could also lead to beneficial behavioral effects that persist into adulthood. This could eliminate or most likely reduce the need for chronic pharmacological treatments in adulthood. Although the

results for the treatment of pediatric ASD patients with bumetanide are encouraging, long-lasting effects of early treatments will have to be addressed first in animal models and then in patients (Grandgeorge et al., 2014; Lemonnier and Ben-Ari, 2010; Lemonnier et al., 2012). Interestingly, the administration of fluoxetine, SAG1.1 or choline during gestation or in the early postnatal period has already demonstrated long-lasting and beneficial effects in the memory of Ts65Dn mice (Ash et al., 2014; Bianchi et al., 2010; Das et al., 2013; Guidi et al., 2013; Moon et al., 2010; Velazquez et al., 2013). Interestingly, besides early pharmacological treatment, the regulation of the CCCs expression by viral injection in parenchyma or through the several other available techniques (Cwetsch et al., 2018) could represent a valuable option to perform an early intervention and achieve long-lasting effects in the brain.

In conclusion, the results obtained from basic research on brain development and from studies on animal models of DS and other ND indicate the NKCC1/KCC2 imbalance as one of the mechanisms underlying brain development deficits and behavioral impairments that characterize ND. Interestingly, the aforementioned studies also suggest that timely treatment with specific NKCC1 inhibitors may lead to positive outcomes. Thus, new drugs and possibly innovative genetic therapeutic approaches selectively targeting CCCs should be tested at different times during development in ND animal models and possibly in clinical trials with the hope that timely and specific intervention on CCCs will ameliorate the symptoms of patients affected by NDs in the future.

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