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KNOCKING OUT THE Na^{+}/Ca^{2+} EXCHANGER NCX3 IMPAIRS OLIGODENDROCYTE LINEAGE RESPONSES AND WORSENS CLINICAL SYMPTOMS IN EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS-INDUCED MULTIPLE SCLEROSIS IN MICE

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SUMMARY

The dysregulation of $[Ca^{2+}]_i$ and $[Na^+]_i$ homeostasis is involved in neuronal and glial response occurring in several neurodegenerative diseases, including Multiple Sclerosis. The Na^+/Ca^{2+} exchanger can be considered a key player in modulating the $[Ca^{2+}]_i$ and $[Na^+]_i$ homeostasis following the injury. Recent evidence point out to the isoform NCX3 of the Na^+/Ca^{2+} exchanger as a new potential therapeutic target for neuroprotection. The aim of the present study was to establish the role played by NCX3 in a murine model of Multiple Sclerosis. The experimental model used in these studies was the Experimental Autoimmune Encephalomyelitis (EAE). Biochemical analysis performed on spinal cord tissue homogenates revealed that NCX3 protein levels were progressively up-regulated during EAE progression; this effect was more significant at EAE chronic stage. In addition, quantitative confocal double immunofluorescence experiments showed that the co-expression of NCX3 with both the myelin marker myelin basic protein (MBP) and the axonal marker neurofilament 200 (NF200) was significantly down-regulated at peak and chronic stages of EAE disease. By contrast, quantification of colocalization studies revealed that the co-expression of NCX3 with the oligodendrocyte lineage markers, the membrane chondroitin sulfate proteoglycan NG2, the Galactocebroside (GalC), and the 2'-3'-cyclic nucleotide-3'-phosphodiesterase (CNPase) was up-regulated during EAE progression. Interestingly, this up-regulation was more significant at EAE chronic stage. These early results suggested that NCX3 isoform might be involved in neuroprotective responses mediated by oligodendrocytes during the EAE recovery phase. The importance of the NCX3 isoform in oligodendroglial responses following EAE insult was supported by several findings: 1) at chronic stage of EAE disease,

NCX3 knockout (ncx3-/-) mice displayed a reduced number of NG2 and CNPase positive cells when compared to NCX3 congenic wild type (ncx3+/+) mice; 2) NCX3 knockout (ncx3-/-) mice showed an earlier onset of symptoms and an increased susceptibility to the EAE disease when compared to NCX3 congenic wild type (ncx3+/+) mice. In conclusion, our findings suggested that NCX3 exchanger, by modulating $[Na^+]_i$ and $[Ca^{2+}]_i$ homeostasis might play an important role in controlling oligodendrocyte response after a demyelinating insult.

I. INTRODUCTION

1. The Na^+/Ca^{2+} Exchanger (NCX)

1.1 The Na^+/Ca^{2+} Exchanger Molecule: an overview

The Na^+/Ca^{2+} exchanger is a transporter catalyzing the countertransport of three Na^+ for one Ca^{2+} and is present in the plasma membrane of most cells (Philipson and Nicoll, 2000). NCX can mediate Ca^{2+} and Na^+ fluxes across the synaptic plasma membrane in a bidirectional way (Blaustein and Lederer, 1999; Philipson and Nicoll, 2000). The Na^+/Ca^{2+} exchanger was discovered and characterized in the late 1960s, when Baker et al. in the UK (Baker and Blaustein, 1968; Baker et al., 1969), Reuter and Seitz in Germany and Switzerland (Reuter and Seitz, 1968), and Martin and De Luca in the United States (Martin and De Luca, 1969) realized the presence of a countertransport mechanism that exchanged Na^+ and Ca^{2+} ions across the plasma membrane of different excitable and non excitable cells. However, the most crucial advancement in NCX research was made in 1988 (Philipson et al., 1988) and 1990 (Nicoll et al., 1990), when Philipson and his colleagues purified and cloned the first isoform of this antiporter: NCX1. Remarkably, four years later, the same group of investigators cloned NCX2 (Li et al.,1994) and NCX3 (Nicoll et al., 1996b), two isoforms selectively expressed in the brain (Lee et al., 1994) and in the skeletal muscle (Nicoll et al., 1996b). Finally, in 1999, Philipson proposed a new topological model of the NCX1 exchanger (Nicoll et al., 1999).

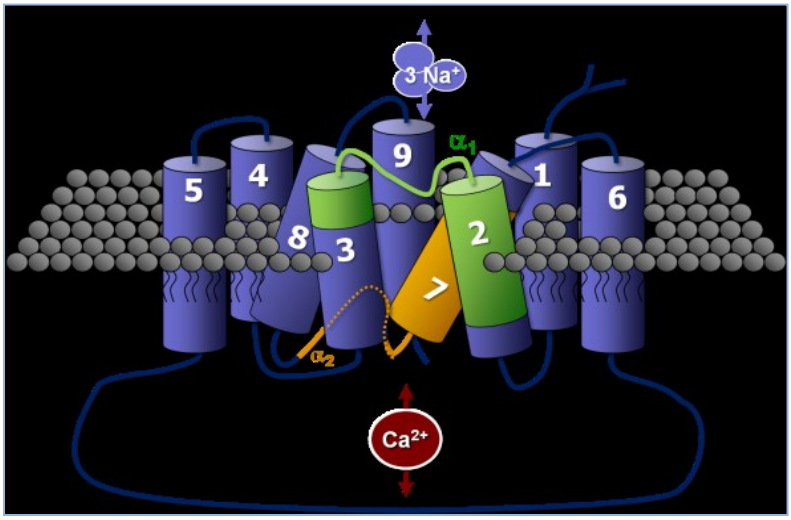
In the last two decades, there has been a growing interest in unraveling the role that the Na^+/Ca^{2+} exchanger plays in the function and regulation of several cellular activities. Biochemistry, molecular biology,

electrophysiology, genetically modified mice, and molecular pharmacology have helped to delve deeper and more successfully into the physiological and pathophysiological role of this exchanger (Annunziato et al., 2004; Philipson et al., 2002).

1.2 Molecular Biology of the Na^+/Ca^{2+} Exchanger

Three dominant genes coding for the three different NCX1 (Nicoll et al.,1990), NCX2 (Li et al., 1994), and NCX3 (Nicoll et al., 1996a) proteins have been identified in mammals. The ncx1 gene displays an ubiquitous expression, whereas NCX2 and NCX3 gene products have been found exclusively in neuronal and skeletal muscle tissues (Lee et al.,1994).These three genes appear to be dispersed, since NCX1, NCX2, and NCX3 have been mapped in mouse chromosomes 17, 7, and 12, respectively (Nicoll et al.,1996b). NCX1 is composed of 938 amino acids having a theoretical molecular mass of 120 kDa. NCX2 and NCX3 gene products consist of 921 and 927 amino acids and are characterized by molecular masses of 102 and 105 KDa, respectively. All three NCX gene products share the same membrane topology. On the structural point of view, NCX is composed by nine transmembrane segments (Nicoll et al.,1999) that can be divided into an N-terminal hydrophobic domain, composed of the first five TMS (1–5), and into a C-terminal hydrophobic domain, composed of the last four TMS (6–9) .The amino terminus domain is located in the extracellular space, whereas the carboxyl terminus domain is located intracellularly. These two hydrophobic domains are important for the binding and the transport of ions. The N-terminal hydrophobic domain (TMS 1–5) is separated from the C-terminal hydrophobic domain (TMS 6–9) through a large hydrophilic intracellular *loop* of 550 amino acids, named the *f loop* (Nicoll et al. 1999). Although the *f loop* is not implicated in Na^+ and

Ca^{2+} translocation, it is responsible for the regulation of NCX activity elicited by several cytoplasmic messengers and transductional mechanisms, such as H^+, Ca^{2+}, and Na^+ ions, nitric oxide (NO), phosphatidylinositol 4,5 bisphosphate (PIP2), protein kinase C (PKC), protein kinase A (PKA), phosphoarginine (PA), and ATP. In the center of the *f loop*, a region of approximately 130 amino acids in length (371–508 amino acids) has been reported to exert a Ca^{2+} regulatory function. This region is characterized by a pair of three aspartyl residues and by a group of four cysteines (Nicoll et al., 1999; Qiu et al., 2001). At the N-terminal end of the *f loop* near the membrane lipid interface, an autoinhibitory domain, rich in both basic and hydrophobic residues and consisting of a 20-amino acid sequence (219–238), named exchange inhibitory peptide (XIP) (Matsuoka et al., 1997), has been identified. The *f loop* is also characterized by alternative splicing sites named β1-repeat and β2-repeat. These β-repeats are characterized by similar regions comprising 60 to 70 amino acids for which no functional role has yet been proposed (Hilgemann, 1990). The NCX protein amino acid sequence found between TMS 2 and TMS 3 is called α-1 repeat, whereas the one found between TMS 7 and TMS 8 is named α-2 repeat. Both regions, α-1 and α-2 repeats, are located on the opposite site of the membrane and include two segments composed of 12 and 9 highly conserved residues separated by a nonconserved segment of 18 to 20 amino acids (Nicoll et al., 2002). Since the putative α-helices of the α-repeats are amphipathic, the hydrophilic faces of these helices may form a portion of the ion translocation pathway (Nicoll et al., 1996a) (Fig. 1).



Adapted from Iwamoto et al., Mol Pharmacol, 2004

Figure 1. Topological model of the Na^+/Ca^{2+} exchanger (NCX) protein. NCX is composed by nine transmembrane segments that can be divided into an N-terminal hydrophobic domain, composed of the first five TMS (1-5), and into a C-terminal hydrophobic domain, composed of the last four TMS (6-9). The N-terminal hydrophobic domain (TMS 1–5) is separated from the C-terminal hydrophobic domain (TMS 6–9) through a large hydrophilic intracellular *loop* of 550 amino acids, named the *f loop*. The conserved α1- and α2-repeat regions which span TMS 2 and 3, and TMS 7 and 8 are highlighted.

1.3 Functional Activity of NCX in Neurophysiological Conditions

The regulation of intracellular concentrations of Ca^{2+} and Na^+ ions in excitable cells is a relevant physiological phenomenon that maintains cellular homeostasis. In fact, although cytosolic Ca^{2+} ions play a key role in signaling at the cytosolic and nuclear levels (Choi, 1988), Na^+ ions play a major role in regulating cellular osmolarity, in inducing action potential, and in transduction signaling (Yu et al., 1997). Indeed, the Na^+/Ca^{2+} exchanger, in parallel with selective ion channels and ATP-dependent pumps, maintains the physiological cytosolic concentrations of these ions (Blaustein and Lederer, 1999). The Na^+/Ca^{2+} exchanger

protein is involved in different neurophysiological conditions. In particular, in the Central Nervous System (CNS), it plays a fundamental role in controlling changes in the intracellular concentrations of Na^+ and Ca^{2+} ions that occur in physiologic conditions such as neurotransmitter release, cell migration and differentiation, gene expression (Annunziato et al., 2004). The Na^+/Ca^{2+} exchanger can mediate Ca^{2+} and Na^+ fluxes across the synaptic plasma membrane in a bidirectional way (Blaustein and Lederer, 1999; Philipson and Nicoll, 2000). The stoichiometry of NCX is generally accepted to be three Na^+ ions/one Ca^{2+} ion. When intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) rise and the cells require the return to resting levels, this exchanger transport mechanism couples the uphill extrusion of Ca^{2+} ions to the entry of Na^+ ions into the cells down their electrochemical gradient. This mode of operation, defined as *forward mode* or Ca^{2+} efflux or, more correctly, Ca^{2+}-exit mechanism, keeps the 104-fold difference in $[Ca^{2+}]_i$ across the cell membrane. In physiological as well in several pathophysiological conditions, when the intracellular Na^+ concentrations ($[Na^+]_i$) rise or membrane depolarization occurs, thus reducing the transmembrane Na^+ electrochemical gradient, NCX reverses its mode of operation and mediates the extrusion of Na^+ and the entry of Ca^{2+} ions. This mode of operation is defined as *reverse mode* or Ca^{2+}-entry mechanism. The mode of operation of the antiporter depends on (1) the transmembrane Na^+ gradient, (2) the transmembrane Ca^{2+} gradient, and (3) the membrane potential.

The Na^+/Ca^{2+} exchanger becomes the dominant Ca^{2+} extrusion mechanism when $[Ca^{2+}]_i$ is higher than 500 nM, as it happens when a train of action potentials reaches the nerve terminals. It has been calculated that for these $[Ca^{2+}]_i$ values (500 nM), more than 60% of Ca^{2+} extrusion is mediated by Na^+/Ca^{2+} exchanger families. In such physiological conditions, NCX activation is consistent with its low-affinity and high-capacity function. In contrast, in resting conditions or after a

single action potential, when $[Ca^{2+}]_i$ slightly increases, requiring, therefore, a more subtle control, the high-affinity and low-capacity pump, plasma membrane Ca^{2+} ATPase, assumes a predominant function, thus making the involvement of NCX less relevant (Blaustein and Lederer, 1999).

Evidence has shown that NCX can modulate not only the synthesis and the release of neurotransmitters in the central and peripheral nervous system but also the release of anterior pituitary hormones (Taglialatela et al.,1988a,b; Di Renzo et al., 1995). It has been shown that NCX is also involved in the cellular events triggered by the activation of G-protein-coupled neurotransmitter receptors. Thus, it has been demonstrated that the serotonin-induced increase of the firing rate of histaminergic tuberomammilar neurons is weakened by NCX inhibitors (Eriksson et al., 2001a,b). Interestingly, the serotonin receptor 5-HT2C, which mediates this effect on histaminergic tuberomammillar neurons, is also co-expressed with NCX1 (Sergeeva et al., 2003). Moreover, in the same histaminergic neurons, NCX activity plays a relevant role in orexin-induced depolarization (Eriksson et al., 2001b). Therefore NCX is a major player in the regulation of physiological responses to increases of $[Ca^{2+}]_i$ and $[Na^+]_i$ (Annunziato et al., 2004; Philipson and Nicoll 2000).

1.4 Genetically Modified-Mice for different NCX Isoforms

In the last three decades an increasing number of compounds inhibiting NCX have been synthesized in order to identify the physiological role played by NCX isoforms, NCX1, NCX2 and NCX3; however, at present no isoform-specific compounds have been yet identified (Annunziato et al., 2004). Moreover, these drugs, despite their potency, possess some

non specific actions against several ion channels and receptors (Reuter et al., 2002a) as a result it is difficult to discriminate the direct effects of NCX inhibition from the inhibition/activation of channel receptors or enzymes. Thus, the use of these non specific inhibitors to characterize the role of each NCX isoform should be interpreted with caution (Reuter et al., 2002a). Because of the limitations of the currently available pharmacological tools acting on NCX, transgenic mice for NCX1, NCX2 and NCX3 were generated in order to identify the role of each antiporter isoform under physiological and pathophysiological conditions. In 2000 four independent laboratories generated knockout mice for NCX1. Unfortunately, these mice die in utero at 9.5 days poistcoitum because the lack of cardial NCX1 in homozygote state causes the absence of a spontaneous heart beat (Cho et al., 2000; Koushik et al., 2001; Reuter et al., 2002b; Wakimoto et al., 2000). Later, Henderson et al. (2004) showed how to over-come this limitation by using the Cre-loxP technique in the heart demonstrating that NCX1 gene ablation in adult mice is compatible with life. As far as the other isoform NCX2 is concerned, its knocking out is compatible with life and survival until adulthood (Jeon et al., 2003). Also NCX3 knockout (ncx3-/-) mice survive until the adulthood. In particular, ncx3 -/- mice were fertile, they appeared grossly normal and showed normal body weight (Sokolow et al., 2004). The use of genetic-modified mice for NCX1, NCX2 and NCX3 represents a fruitful strategy to characterize the physiological role exerted by NCX in CNS and to identify the isoforms of the antiporter as potential molecular targets for therapeutic intervention.

1.5 Distribution of Na^+/Ca^{2+} Exchanger Isoforms

The distribution of the three NCX isoforms in the mammalian brain gave useful insights into the physiological and pathophysiological role played

by the different NCX isoforms in the regulation of neuronal function. In fact, NCX1, NCX2 and NCX3 are highly and selectively expressed in the mammalian brain, suggesting that NCX proteins may play a differential role in the regulation of brain cell functions. Indeed, NCX1 and NCX3 have several splicing variants that appear to be selectively expressed in different regions and cellular populations of the brain (Quednau BD et al., 1997; Yu SP and Choi DW, 1997). The NCX1 gene displays an ubiquitous expression and therefore is present in several tissues, including brain, heart, skeletal muscle, smooth muscle, kidney, eye, secretory, and blood cells whereas NCX2 and NCX3 gene products have been found exclusively in neuronal and skeletal muscle tissues (Lytton J, 2002). In the cerebral cortex, NCX1 is intensively expressed in neurons of layer III and V within the molecular layer of the cerebral motor cortex. This area, which contains the terminal dendritic field of the pyramidal cells, displays a more intense NCX1 immunoreactivity than that of NCX2. In contrast, the somatosensory cortical area seems to express preferentially NCX2 transcripts. Such anatomical distribution reveals that the upper neurons of the motor system and the terminal neurons of the somatosensory system preferentially express distinct NCX isoforms (Canitano et al., 2002; Papa et al., 2003). Within the hippocampus, the transcripts of the three NCX isoforms display an intense labeling within selective neuronal populations. In particular, high levels of the three NCX genes have been detected in the granular cell layers of the dentate gyrus and in the pyramidal cells of CA1, CA2, CA3, and CA4 subfields (Papa et al., 2003). The three NCX protein isoforms also display high levels of expression within the hippocampus. Thus, in the oriens and radiatum layers of the CA1 subfield, NCX3 protein is more intense than NCX1 and NCX2. NCX1 protein expression is particularly intense in the granule cell layer and in the hilus of the dentate gyrus, which constitutes

the terminal field of the perforant pathway, the major excitatory input to the hippocampus originating from the enthorinal cortex. In the CA3 area, the expression of NCX1 and NCX3 genes within the mossy fibers projecting from the granule cells located in the dentate gyrus are more intense than those of NCX2. This peculiar distribution suggests that distinct NCX isoforms may play a crucial role in controlling the intracellular Na^+ and Ca^{2+} homeostasis of the major afferent, intrinsic, and efferent hippocampal projections. Such circuitries are crucial for the synaptic plasticity phenomena, such as those involved in long-term potentiation (LTP) and long-term depression (Madison et al.,1991). NCX isoforms are also expressed in crucial areas of the extrapyramidal control of motor coordination. In fact, NCX1 mRNA can be detected in the substantia nigra pars compacta, in which dopaminergic cell bodies are localized; the NCX1 protein isoform is present in the striatum, in which the terminal projection fields of dopaminergic nigrostriatal neurons are found. Interestingly, both the transcripts and the proteins, encoded by the three NCX genes, are abundantly expressed in the nucleus accumbens (Canitano et al., 2002; Papa et al.,2003).

1.6 The Na^+/Ca^{2+} Exchanger in Glial Cells

The Na^+/Ca^{2+} exchanger protein is present in both neurons and glia, and may play a relevant function in different neurophysiological conditions. In glial cells the Na^+/Ca^{2+} exchanger is significantly expressed (Finkbeiner, 1993; Goldman et al., 1994; Holgado and Beauge, 1996; Takuma et al., 1994) but its precise role has not yet been determined. Glial cells constitute the large majority of cells in the nervous system. They account for about 90% of all cells in the human brain. During recent years a large number of studies have critically changed the image of glia from a silent and passive supportive cells to

important partner of neurons in brain functions. Glial cells are involved in almost every type of neurodegenerative diseases. Alteration of intracellular ionic homeostasis in response to CNS injury plays a crucial role in inducing and maintaining glial responses in the injured brain. Reactions of glial cells which include the reaction of astroglia, such as reactive astrogliosis, microglia, such as activation of microglia, and oligodendroglia, such as Wallerian degeneration and demyelination are of critical importance for the progress of neural pathology (Annunziato et al., 2013).

1.6.1 NCX in Microglia

All three NCX exchangers, NCX1, NCX2 and NCX3 are expressed in microglial cells, but NCX1 is the most highly expressed (Nagano T et al., 2004; Boscia et al., 2009). NCX activity in microglia was first recorded by Matsuda et al. (2001) who observed a Na^+-dependent $45Ca^{2+}$ uptake inhibited by the NCX inhibitor SEA0400. Functional studies indicated that NCX1 in microglia plays a key role in the response to IFN-γ and NO, two cell products implicated in several CNS pathologies including stroke. Treatment of microglial cells with interferon-γ caused a biphasic increase in NCX activity. The delayed increase in NCX activity was accompanied by increases in mRNA and protein levels of all NCX isoforms (Nagano T et al., 2005). Pharmacological studies show that protein kinase C and tyrosine kinase inhibitors prevent the transient increase in NCX activity induced by IFN-γ, whereas a mitogen-activated protein (MAP) kinase/extracellular signal regulated protein kinase (ERK) is involved in the delayed increase in NCX activity (Nagano et al., 2004). Furthermore, using the specific NCX inhibitor SEA0400, Nagano et al. (2005) showed that NO

activates NCX and that NCX is involved in NO-induced depletion of Ca^{2+} in the endoplasmic reticulum (ER), leading to ER stress. The responses of NCX to interferon-γ and NO implies that Ca^{2+} uptake via NCX operating in reverse mode may play a role for microglial activation under pathological conditions. Recently, a prominent role of NCX in microglial migration have been suggested (Ifuku et al., 2007). In fact, Ca^{2+} influx via reverse-mode activity of the Na^{+}/Ca^{2+} exchanger is prerequisite for bradykinin and B1 receptor agonist-induced microglial motility. The knocking out of NCX1 in the heterozygous state impairs bradykinin-induced chemotaxis or migration in microglia. The relevance of NCX1 function in microglia are further supported by the findings that ncx1-/- embryos have no detectable microglia in the brain when compared to ncx1+/+ control littermates (Ginhoux et al., 2010). The crucial role of NCX1 isoform in activated microglia under patophysiological conditions is becoming further evident. Phagocytosis and the ensuing NADPH-mediated respiratory burst are important aspects of microglial activation that require calcium influx. Inhibiting the reverse mode of the Na^{+}/Ca^{2+} exchanger with KB-R7943, dose dependently reduced the phagocytosis-stimulated respiratory burst (Newell et al., 2007). In accordance with the role played by NCX in phagocytic microglia, it has been demonstrated that 3 and 7 days after pMCAO, NCX1 signal progressively increased in the Iba1-positive microglia invading the infarct core. In these cells, NCX1 expression was limited to the round phagocytic phenotype, which represents the final stage of microglia activation (Boscia et al., 2009).

1.6.2 NCX in Astrocytes

The Na^{+}/Ca^{2+} exchanger is abundantly expressed in astrocytes (Finkbeiner, 1993; Goldman et al., 1994; Holgado and Beauge, 1996;

Takuma et al., 1994). In particular, NCX1 represents the most abundant NCX isoform expressed in astrocytes (He et al., 1998), where, when operating in the reverse mode, it can deliver Ca^{2+} to the cytosol (Kirischuk et al., 1997; Rojas et al., 2008). The most important role played by NCX1 in astrocytes is related to the regulation of gliotransmitter release (Zorec et al., 2012). Hence, mild depolarization of astrocytes cultured from adult rats forces NCX to operate in the reverse mode and generates cytosolic Ca^{2+} increases leading to release of glio-transmitters such as glutamate (Paluzzi et al., 2007; Reyes and Parpura, 2009). On the other hand, it should be underlined that since the gliotransmitter release is modulated by cytosolic Ca^{2+} levels in astrocytes, NCX1 is not the only protein involved in this process. In fact, mitochondria (Duchen et al., 2008), SERCA and Ca^{2+}-ATPase (Reyes et al., 2012), by regulating cytosolic Ca^{2+} levels, trigger exocytotic release of different gliotransmitters from astrocytes (Parpura and Zorec, 2010).

1.6.3 NCX in Oligodendrocytes

All three NCX isoforms have been detected in primary cultures of oligodendrocytes and oligodendrocyte precursor MO3.13 cell line (Quednau et al., 1997; Boscia et al., 2012). The staining of NCX1 in myelinated axons of the sciatic nerve, optic nerve and spinal cord, is similar being associated both with axons as well as oligodendrocyte cell bodies and their processes. The lack of NCX staining in some white matter regions, as NCX1 in the corpus callosum, raises the possibility that the localization of the three exchanger isoforms may be different within white matter tracts (Steffensen et al., 1997). NCX1 and NCX3 isoforms are differently expressed in Oligodendrocyte Precursor Cells

(OPCs) and are divergently modulated during differentiation of OPCs into oligodendrocytes (Boscia et al., 2012). Indeed, whereas NCX1 isoform decreases, NCX3 isoform is strongly increased during oligodendrocyte maturation. Expression and functional studies suggest that NCX1 represents the main contributor to NCX currents (I_{NCX}) recorded in OPCs. Tong et al. (2009) have shown that NCX1, but not NCX2, is highly expressed in OPCs and that pharmacological inhibition of NCX1 or its selective silencing with siRNA largely inhibits migration and GABA-induced Ca^{2+} signaling in cultured OPCs. NCX3 represents the main isoform expressed in mature oligodendrocytes and, consequently, the main contributor to I_{NCX} recorded in these cells. The importance of calcium signaling mediated by NCX3 exchanger during oligodendrocyte development and myelin formation is supported by several findings: 1) the knocking down of NCX3 expression and activity by siRNA strategy in OPC cultures prevented the up-regulation of the myelin proteins; 2) NCX3 overexpression induced the up-regulation of the two myelin markers CNPase and MBP; 3) NCX3 knockout mice exhibit hypomyelination that is accompanied by an augmented number of the OPC cells, and a reduction of spinal cord size (Boscia et al., 2012). Previous studies have demonstrated a central role of NCX in anoxic and ischemic injury of both central and peripheral myelinated axons. Indeed, the reverse mode operation of NCX has been implicated in axonal damage during spinal cord anoxia (Li et al., 2000), stretch injury of axons (Wolf et al., 2001), and experimental allergic encephalomyelitis (Craner et al., 2004). In oligodendrocytes NCX1 and NCX3 are co-localized with the Na^+-K^+-$2Cl^-$ co-transporter (NKCC1). The reverse mode operation of NCX and NKCC1 has been also implicated in oligodendrocyte cell death induced by AMPA-mediated excitotoxicity. Activation of AMPA receptors leads to NKCC1 phosphorylation that enhances NKCC1-mediated Na^+ influx. The latter

triggers NCX in the reverse mode of operation with consequent Ca^{2+} overload that compromises mitochondrial function and cellular viability (Chen et al., 2007).

1.7 Relevance of Na^+/Ca^{2+} Exchanger Activity in Neurodegenerative Conditions

The dysregulation of $[Ca^{2+}]_i$ and $[Na^+]_i$ homeostasis is involved in neuronal and glial injury occurring in *in vitro* and *in vivo* models of several neurodegenerative diseases (Annunziato et al., 2004). The Na^+/Ca^{2+} exchanger can be considered a major player in the regulation of neuropathophysiological responses following alterations in $[Ca^{2+}]_i$ and $[Na^+]_i$ (Annunziato et al., 2004; Philipson and Nicoll 2000).

1.7.1 Hypoxia-Anoxia

Cell calcium is thought to play a key role in mediating ischemic neuronal damage (Kristian and Siesjo, 1996), and thus it is critical to understand how it is regulated. Cytosolic Ca^{2+} is elevated during global ischemia (Erecinska and Silver, 1992; Nakamura et al., 1999) and during *in vitro* ischemia (deprivation of glucose and oxygen) in brain slices (Mitani et al., 1993), as well as in neuronal cell cultures that are exposed to mitochondrial and glycolytic inhibitors (Dubinsky and Rothman, 1991). Ischemia induces a large increase in glutamate release in brain tissue *in vivo* (Benveniste et al., 1984) and *in vitro* (Lobner and Lipton, 1990), and there is evidence of an NMDA component to the increase in cytosolic Ca^{2+} during global ischemia (Erecinska and Silver, 1992), during anoxia in cortical slices (Bickler and Hansen, 1994), and in organotypic hippocampal cultures (Velazquez et al., 1997). The Na^+/Ca^{2+} exchanger represents a key mediator for maintaining

intracellular $[Na^+]_i$ and $[Ca^{2+}]_i$ homeostasis in response to brain ischemia (Boscia et al., 2009). Nevertheless, the role played by NCX proteins in cell injury in different experimental models of cerebral ischemia remains controversial. In a cellular model of glial cells, C6 glioma, the activation of NCX, as Na^+-efflux Ca^{2+}-influx pathway, obtained by $[Na^+]_e$ removal, reduces cell injury induced by chemical hypoxia. Such phenomenon suggests that the antiporter plays a protective role during this pathophysiological condition. Consistent with these results, the pharmacological inhibition of NCX activity worsens cell damage by increasing the intracellular concentration of Na^+ ions (Amoroso et al., 1997). Furthermore, the stimulation of NCX activity by redox agents results in a protective effect (Amoroso et al., 2000). In addition, in cells singly and stably transfected with NCX3, this isoform contributes more significantly to the maintenance of $[Ca^{2+}]_i$ homeostasis during experimental conditions mimicking ischemia, thereby preventing mitochondrial $\Delta\Psi$ collapse and cell death (Secondo et al., 2007). Consistent with the protective function played by NCX, in cortical neurons exposed to oxygen and glucose deprivation, the nuclear factor kappa-B-dependent NCX1 up-regulation seems to play a fundamental role in Ca^{2+} refilling in the endoplasmic reticulum, thus helping neurons to prevent endoplasmic reticulum stress (Sirabella et al., 2009). Inconsistent with these results, in astrocytes, during the reoxygenation phase, the inhibition of the antiporter decreases cell toxicity (Matsuda et al., 1996, 2001), whereas NCX stimulators, such as NO donors, worsen the injury (Matsuda et al., 1996). Conflicting reports on the role played by NCX activity in glutamate-induced neuronal damage, an *in vitro* model mimicking cerebral ischemia, have been published (Andreeva et al., 1991; Kiedrowski et al., 1999; Schroder et al., 1999; Amoroso et al., 1993). For instance, studies on cerebellar granule cells, as well as on glial cells (Amoroso et al., 2000), have reported that the inhibition of

NCX exacerbates delayed neuronal death elicited by glutamate (Andreeva et al., 1991). In contrast, in the same model, other investigators have demonstrated that Ca^{2+} influx, mediated via reverse mode of NCX operation, constitutes the dominant component of NMDA-induced Ca^{2+} entry and excitotoxicity (Kiedrowski, 1999). The same mechanism, an Na^{+}-mediated reversal of NCX activity, leads to the death of depolarized and glucose-deprived neurons (Czyz and Kiedrowski, 2002). In line with the latter hypothesis, it has been demonstrated that in rat hippocampal slices, the inhibition of NCX protects CA1 neurons against hypoxic-hypoglycemic injury (Schroder et al., 1999). In *in vivo* models, reproducing human cerebral ischemia through the occlusion of the middle cerebral artery, the inhibition of NCX, induced by selective inhibitors (Pignataro et al., 2004) or by the knockout of one of the NCX isoforms (NCX2) (Jeon et al., 2003a), aggravates brain infarct, whereas the activation of the antiporter with redox agents reduces the cerebral infarctual area (Pignataro et al., 2004b). In addition, in *in vivo* experiments, the selective knocking down of NCX1 and NCX3, but not of NCX2, by antisense oligodeoxynucleotide strategy (Pignataro et al., 2004), or the disruption of the ncx3 gene, renders the brain more susceptible to the ischemic insult (Molinaro et al., 2008). At variance with these data, Matsuda et al. (2001) reported that the inhibition of NCX, induced by putative selective NCX inhibitors, such as KB-R7943 and SEA0400, reduces brain injury in the model of transient middle cerebral artery occlusion. However, KB-R7943, besides blocking the antiporter, also produces a remarkable and prolonged hypothermic effect (Pignataro et al., 2004b) that exerts, by itself, a relevant neuroprotective action in cerebral ischemia. On the other hand, by inhibiting other cellular ionic transport mechanisms and receptors, such as NMDA receptors and L-type Ca^{2+} channels (Matsuda et al., 2001), the same drug may yield a neuroprotective effect (Lo et

al., 2003). In regard to the other putative NCX inhibitor, SEA0400, it should be underlined that Matsuda et al. (2001) used an animal model of cerebral ischemia in which the neuronal damage mainly occurred during the reperfusion period with a Ca^{2+} paradox-like injury accompanied by an exaggerated ROS production and apoptotic cell death (Matsuda et al., 2001). These data suggest that if NCX proteins are neuroprotective during ischemic injury, the major role is prevalently exerted by the NCX1 and NCX3 gene products. Taken togheter, at present, it is not clear whether the function of NCX exchangers mediate positive or negative effects after ischemic episodes. A new emerging concept is that NCX1, NCX2 and NCX3 may exert different roles during *in vitro* (Secondo et al. 2007) and *in vivo* (Annunziato et al 2004) anoxic conditions. Consistent with the idea of a differential function for the three NCX proteins, their pathophysiological implications may as well be distinct. The role played by NCX in those neurons and glial cells involved in cerebral ischemia should be differentiated according to the anatomical regions involved in the ischemic pathological process. In particular, it is conceivable that, since in the penumbral region ATPase activity is still preserved, NCX may likely operate in a forward mode. As a result, by extruding Ca^{2+} ions, the exchanger favors the entry of Na^{+} ions. Therefore, the inhibition of NCX in this area reduces the extrusion of Ca^{2+} ions, thus enhancing Ca^{2+}-mediated cell injury. In contrast, in the ischemic core region, in which ATP levels are remarkably low and Na^{+}/K^{+} ATPase activity is reduced, intracellular Na^{+} ions massively accumulate because of Na^{+}/K^{+} ATPase failure. Hence, the intracellular Na^{+} loading promotes NCX to operate in the reverse mode as an Na^{+} efflux-Ca^{2+} influx pathway. In conclusion, the NCX pharmacological inhibition in this core region further worsens the necrotic lesion of the surviving glial and neuronal cells as the loading of intracellular Na^{+} increases (Pignataro et al., 2004b).

1.7.2 Aging

The impairment of Ca^{2+} homeostasis in neuronal cells is considered to be the major triggering event that leads to the development of brain aging (Annunziato et al., 2002). Studies performed on the cerebro-cortex nerve endings of aged rats have shown that the activity of NCX is markedly reduced in the forward and in the reverse mode of action (Michaelis et al., 1984; Canzoniero et al., 1992). NCX decline seems to be the consequence of a reduced affinity of the antiporter for Ca^{2+} ions (Michaelis et al., 1984).

1.7.3 Alzheimer's Disease

A large bulk of studies have shown that the neurotoxicity exerted by the amyloid-β (A-β) protein is intimately related to intracellular Ca^{2+} concentrations. Indeed, the attenuation of $[Ca^{2+}]_i$ increase by Ca^{2+} channel blockers (Weiss et al., 1994), growth factors (Mattson et al., 1993), and cytochalasins (Furukawa and Mattson, 1995) results in a reduction of neural damage induced by the A-β protein. It has recently been demonstrated that exposure to the A-β protein partially reduces Na^+-dependent Ca^{2+} accumulation in plasma membrane vesicles deriving from the human frontal cortex of patients affected by Alzheimer's disease (Wu et al., 1997). These findings have suggested that A-β directly interacts with the hydrophobic surface of the NCX molecule, thus interfering with plasma membrane Ca^{2+} transport.

Sokolow et al., 2011 demonstrated that high levels of synaptosomal Na^+-Ca^{2+} exchangers (NCX1, NCX2, NCX3) co-localized with amyloid-beta in human cerebral cortex affected by Alzheimer's disease. More recently, it has been shown that Aβ(1-42), through Ca^{2+}-dependent calpain activation, generates a hyperfunctional form of NCX3 that, by

increasing Ca^{2+} content into ER, delays caspase-12 activation and thus neuronal death (Pannaccione et al., 2012).

In addition, Atherton et al., 2014 demonstrated that calpain cleavage and inactivation of the sodium calcium exchanger-3 occur downstream of Aβ in Alzheimer's disease.

1.7.4 Multiple Sclerosis

Although demyelination and inflammation have classically been considered to be histopathological hallmarks of Multiple Sclerosis, axonal pathology in Multiple Sclerosis has been recognized for over a century (Charcot, 1868). It has recently been demonstrated that axonal degeneration contributes to the development of non-remitting neurological deficits and disability in Multiple Sclerosis, but the molecular mechanisms that underlie axonal loss in Multiple Sclerosis are not clearly understood.

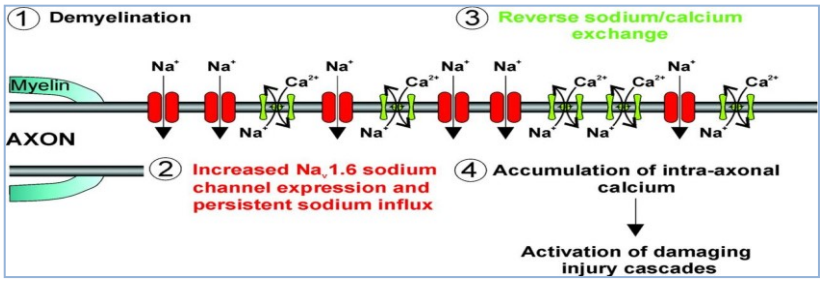
The precise role of the Na^+/Ca^{2+} exchanger in axonal degeneration has not been resolved. Evidence suggesting that voltage-gated sodium channels and the Na^+/Ca^{2+} exchanger might cooperate in triggering degeneration of white matter axons was provided by early studies that demonstrated that sodium channels can participate in the production of calcium-mediated axonal degeneration of axons within the anoxic optic nerve (Stys et al., 1992). Studies of white matter axonal injury have demonstrated that voltage-gated sodium channels can provide a route for sodium influx into axons that triggers reverse operation of the NCX and subsequent influx of damaging levels of intra-axonal calcium. Several reports suggest that NCX plays a major role in mediating Ca^{2+}-induced white matter damage induced by anoxia or trauma. During these noxious stimuli, myelinated axons lose K^+, whereas intra-axonal Na^+ concentrations increase after Na^+ entry, primarily through voltage-

gated tetrodotoxin-sensitive channels. Hence, elevated axoplasmic Na^+ and axolemmal depolarization promote a neurodetrimental Ca^{2+} overload mediated primarily by NCX operating in the reverse mode (Stys and Lopachin, 1998). In fact, drugs capable of inhibiting NCX activity, such as bepridil and amiloride derivatives, reduce white matter damage in different experimental models of axonal injury: rat optic nerve anoxia (Stys et al., 1990; Stys and Lopachin, 1998), spinal cord injury (Li et al., 2000), and stretch-injured axons (Wolf et al., 2001). More recently, a role of NCX and sodium channels in axonal degeneration in neuroinflammatory disorders has been suggested by the protective effect of low doses of the sodium channel blockers lidocaine and flecainide and tetrodotoxin (TTX) (Garthwaite et al., 2002) and the NCX blocker bepridil on axonal injury triggered by nitric oxide (NO).

In addition, Waxam and co-workers (Craner et al., 2004a) have shown that NCX co-localizes with $Na_v1.6$ in the injured axons in the spinal cord of mice with EAE (Craner et al., 2004b). In particular, Craner et al. (2004) have demonstrated the up-regulated expression of $Na_v1.2$ and $Na_v1.6$ along extensive regions of demyelinated axons in EAE. Although the development of a diffuse distribution of $Na_v1.6$ throughout long regions of demyelinated axons may facilitate restoration of action potential propagation (Bostock and Sears, 1976, 1978; Foster et al., 1980; Black et al., 2002), ectopic expression of $Na_v1.6$ along the axonal membrane might also have a deleterious effect on axons, especially if $Na_v1.6$ and NCX are co-localized. Several lines of evidence suggest that $Na_v1.6$ contributes to the persistent current that drives reverse NCX in injured axons in Multiple Sclerosis (Craner et al., 2004). The proposed mechanism involves massive sodium influx into axons that triggers reverse operation of the exchanger and subsequent influx of damaging levels of intra-axonal calcium that is associated with axonal

injury (Craner et al., 2004) (Fig. 2). Overall, these studies emphasize the relevance of NCX modulation in an attempt to prevent white matter degeneration in different models of axonal injury.

In Multiple Sclerosis, it is clear that neurodegenerative processes are linked to irreversible disability in later stages of the disease. In this regard, the role and contribution of calcium pumps and exchangers to Multiple Sclerosis pathology warrant further investigations.



Adapted from Craner et al., PNAS , 2004

Figure 2. Proposed mechanism of axonal injury by means of co-expression of Nav1.6 and NCX. The model suggests that Nav1.6 sodium channels are up-regulated (1) and expressed along some demyelinated axons, where they produce persistent sodium current (2). The persistent sodium current can drive reverse sodium/calcium exchange (3) and accumulation of intra-axonal calcium (4), triggering injurious secondary cascades and axonal injury.

2. Multiple Sclerosis (MS)

2.1 Introduction to MS

Multiple Sclerosis has been known through past centuries as *paraplegia* (Murray, 2009). The depiction of "*a remarkable lesion of the spinal cord accompanied with atrophy*" by Robert Carswell in 1838 anticipated a more or less complete description of the pathological anatomy and clinical features of MS. Previously unrecognized, MS makes a fleeting appearance in the early 19th century before taking centre stage as clinical neurology began to flourish in the 1860s. By the beginning of the 20th century, a disease only a few years earlier meriting individual case

reports had become one of the most common reason for admission to a neurological ward. The first classical description of MS was made by Jean Martin Charcot (Charcot, 1868), who also gave this disease a name. It was also Charcot who, in 1877, realised the role of disrupted myelin in the pathogenesis of the disease.

MS is an autoimmune inflammatory demyelinating disease of the CNS, which culminates in progressive neurological deterioration. The ideas developed for the cause and pathogenesis of the disease are based on studies of epidemiology, genetics, pathology, immunology, and neurobiology.

2.2 Epidemiology

MS is the most common inflammatory demyelinating disease of the CNS and the most common disabling neurological disease afflicting young adults. The mean age of onset is approximately 30 years. Almost 70% of patients manifest symptoms between ages 20 and 40. Disease onset rarely occurs prior to 10 or after 60 years of age. There is clear gender difference with females being more frequently affected than men.

The worldwide incidence and prevalence of MS is highly variable with both genetic and geographical factors playing a role. The greatest incidence tends to be at the extremes of latitude in both Northern and Southern hemispheres. The disease is more common in subjects with Western European ancestry. In particular, Scotland and the Outer Hebrides are the regions with the highest recorded prevalence rates of up to 300 cases per 100.000. In England and Wales, prevalence rates have varied from 74 to 112 per 100.000. In contrast, there are ethnic groups that seem to be resistant to the disease despite inhabiting areas of relatively high prevalence rate such as Maoris in New Zealand,

Hutterites and the Natives in Western Canada. There is no clear explanation for the differences in worldwide distribution of MS. Genetic factors seem to be strongly associated. Environmental factors undoubtedly have strong impact on disease distribution and prevalence. This is supported by observations that migration from high-risk to low-risk areas before puberty affords some protection from acquiring MS and numerous outbreaks or clusters of the disease have been described (Kondar et al., 2010).

2.3 Aetiology

The aetiology of MS remains elusive, as both genetic predisposition and environmental factors are indicated. Arguing the merits of one faction versus the other is unproductive. Each is clearly implicated, together with the cultural condition of age at which the interplay between genes and the environment occurs.

The importance of genetic predisposition is evident from very high concordance of the disease occurrence between monozygotic twins, whereas the environmental factors are implicated by the existence of geographical areas with remarkable differences is MS prevalence. The general theory regards MS aetiology as an infection which presents the immune system with an antigen similar to CNS myelin. Numerous viruses that display homology to myelin components are implicated and proposed as the infective trigger, such as hepatitis B virus and the herpes viruses, Epstein-Barr virus, herpes simplex virus, and cytomegalovirus (CMV), as well as influenza viruses and papillomaviruses. The resulting antibodies eventually attack CNS myelin and cause demyelination. This theory, however, is very broad and imprecise, as MS can cover several aetiologically distinct diseases

with similar pathological endpoint and clinical features (Verkharatsky and Butt, 2013).

2.4 Classification and Clinical Course

Clinically, MS manifests itself as a neurological deficits that frequently exhibit a relapsing and remitting pattern and can resolve completely or leave residual deficits. The deficits can involve any part of the CNS alone or in combination. Somatosensory, pyramidal-motor and visual manifestations, the latter due either to inflammatory demyelination in the afferent visual pathways (optic neuritis) or in the efferent visual pathways are among the most common manifestations. In terms of the clinical course, there are several MS subtypes: relapsing-remitting MS (RRMS), secondary progressive (SPMS), primary progressive (PPMS) and progressive-relapsing MS (PRMS) (Lublin and Reingold,1996).

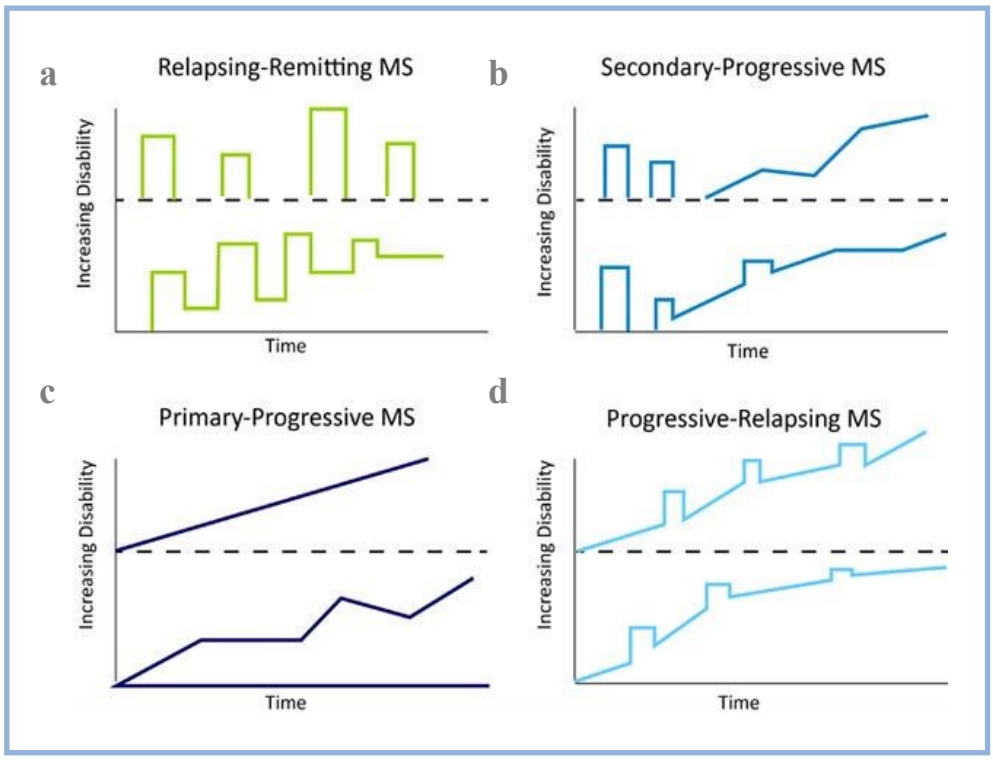
The most common form of the disease is RRMS, which is observed in about 85% of all patients. RRMS is characterized by clearly defined disease relapses with full recovery or with sequelae and residual deficit upon recovery. Although RRMS is not classified as a progressive form of multiple sclerosis, residual deficits may occur after each exacerbation.

At least half of all patients with RRMS will develop to secondary progressive multiple sclerosis (SPMS). This subform is characterize by disease progression with or without occasional relapses, minor remissions, and periods of stability.

In contrast, PPMS is seen in far fewer patients (about 10%). It is characterized from the outset by the absence of acute attacks but demonstrates a worsening in disease severity.

Progressive relapsing multiple sclerosis (PRMS) is the least common form of the disease, affecting about 5% of patients. From the outset it is

progressive, with or without full recovery, and progression is continuous between relapse periods (Fig.3) (Thomson, 2006; Constantinescu et al., 2011).



(Adapted from Lublin and Reingold, Neurology,1996)

Figure 3. The patterns and courses of MS. There are four types of MS: Relapsing-Remitting MS (RRMS), Secondary Progressive MS (SPMS), Primary Progressive MS (PPMS), and Progressive-Relapsing MS (PRMS). **a.** RRMS is characterized by acute attacks with full recovery or with partial recovery. Approximately 80% of patients with MS have RRMS, which is characterized by episodes of symptoms (relapse) followed by the absence of symptoms (remission). Symptoms may evolve over days and disappear, although this varies between patients, and relapse appears approximately every two years. **b.** SPMS occurs as a result of RRMS developing into a progressive state. In this case, the patient previously would have been diagnosed with RRMS, but is now presenting with fewer relapses, with continual progression of neurologic symptoms. **c.** PPMS is characterized by progression from onset of disease without acute relapses. PPMS occurs when the patient's symptoms steadily increase proportional with time, and there are no episodes of relapse or remission. This form of MS has short periods where symptoms stabilize or improve slightly. **d**. PRMS is characterized by progression from onset of disease with acute relapses. PRMS is the rarest form of MS, which is expressed as gradual worsening with superimposed relapse/remission episodes.

2.5 Pathological Features

The pathology of MS is distinguished from that of other inflammatory disease of the nervous system by the presence of large, multifocal, demyelinated plaques or lesions (Lassmann, 1998; Prineas, 1985). There are four key pathological features of MS: (a) *demyelination*; (b) *inflammation;* (c) *axonal loss* or damage; and (d) *gliosis* (Constantinescu et al., 2011).

(a) **Demyelination.** The major pathological hallmark of MS is demyelination. Demyelination is the pathological process in which myelin sheaths are lost from around axons. In the CNS demyelination is usually the consequence of a direct insult targeted at the oligodendrocyte, the cells that makes and maintains the myelin sheath (Franklin and ffrench-Constant, 2008).

(b) **Inflammation.** During active disease, demyelination is associated with an inflammatory reaction that is orchestrated by activated lymphocytes, macrophages, and endogenous glial cells (astrocytes and microglia). Inflammation is generally believed to be the main trigger of the events leading to CNS tissue damage.

However, recent evidence suggests that in some cases initial damage to neuroglia elements can trigger secondary inflammation (Barnett and Prineas, 2004).

Early MS lesions are characterized by the local accumulation of activated $CD4^+$ and $CD8^+$ T-cells around small venules, with $CD4^+$ cells predominating (Hauser et al., 1983). Later there is myelin degeneration associated with perivascular inflammation consisting of T-cells, B-cells, plasma cells and activated macrophages (Prineas, 1975). Macrophage/microglia are active participants in myelin breakdown; phagocytosis of myelin proteins in the lesions by

these cells is a reliable indicator of ongoing demyelinating activity (Bauer et al., 1994).

Based upon pattern and extent of inflammation, white matter lesions are usually divided into active, chronic active, or chronic inactive (Bo et al., 1994; Trapp et al.,1998; van der Valk and De Groot, 2000).

In active lesions, there is macrophage infiltration throughout the lesion; in chronic active lesions, there is macrophage infiltration at the lesion border but little infiltration at the lesion center; and in chronic inactive lesions, there is little infiltration throughout the lesion. In chronic MS, chronic active lesions are the most common lesion subtype in the brain, whereas most of the lesions in the spinal cord are inactive (Tallantyre et al., 2009).

The chronic persistence of inflammation in MS lesions may be clinically relevant as it may mediate the lesion growth and the chronic axonal loss.

(c) **Axonal loss.** Although myelin sheaths are the primary target of tissue destruction, axons are also affected. Acute axonal injury is frequent in actively demyelinating MS lesions. Demyelination can be repaired, at least in part, by remyelination, but axonal destruction is irreversible (Kornek and Lassmann, 1999).

Axonal injury in MS plaques occurs in two stages (Kornek et al., 2000). A high incidence of acute axonal injury is found in lesions during the active stage of myelin destruction. The extent of ongoing axonal loss correlates with extent of macrophage infiltration (Trapp et al., 1998; Ferguson et al., 1997). Thus, even during the earliest stages of the disease, every newly formed plaque is associated with a significant loss of axons. In addition to axonal degeneration in active plaques, there is also a low level of continuous axonal

destruction and loss in chronic inactive demyelinating plaques (Kornek et al., 2000).

(d) **Gliosis.** Gliosis is also a prominent feature of MS, and is characterized by astrocyte proliferation, hypertrophy, and increased synthesis of glial fibrillary acidic protein, an astrocyte-specific protein (Bignami et al.,1972). This reaction is thought to contribute to the formation of dense glial scars in the CNS, leading to motor and sensory impairment.

2.6 Immunology

The pathogenesis of MS is determined by the interaction between multiple components of the immune system and all elements of the CNS (Constantinescu et al., 2001). Following the description of MS by Charcot in 1868 (Charcot, 1868), and the observation by Pasteur at the turn to the twentieth century (Remlinger, 1905) of acute postvaccinal encephalomyelitis in rabies vaccines; Rivers showed in 1933 (Rivers et al., 1933) that the injection of spinal cord or brain homogenates into healthy primates caused a disease similar to MS, leading to the hypothesis that MS is an autoimmune disease. Several decades later, investigators began to study systematically the experimental disease in rodents and made the seminal observations that still dominate our thinking about the pathogenesis of MS (Zamvil et al., 1990). They showed that the injection of defined protein components of the myelin sheath together with an adjuvant into naive susceptible animals caused either an acute, chronic, or relapsing-remitting encephalomyelitis, which is now referred to as Experimental Autoimmune Encephalomyelitis (EAE). However, current evidence on the induction and perpetuation of MS still favors $CD4^+$ autoreactive T cells as a central factor for the autoimmune pathogenesis of MS.

MS is considered a $CD4^+$ Th1-mediated autoimmune disease (Martin et al.,1992; Hafler, 2004). This view is based on the cellular composition of brain- and cerebrospinal fluid (CSF)-infiltrating cells.

The activation of $CD4^+$ autoreactive T cells and their differentiation into a Th1 phenotype is a crucial event in the initial steps of MS, and these cells are probably also important players in the long-term evolution of the disease. Potentially autoreactive $CD4^+$ T cells are activated in the periphery by recognizing, for example, a viral peptide in the context of costimulatory and other less-defined signals. The initiating antigen epitopes show molecular similarity (mimicry) with some CNS antigen or a superantigen (Sospedra and Martin, 2005). Following activation, the effector $CD4^+$ T cells are capable of producing inflammatory cytokines (McFarlard and Martin, 2007).

Factors that contribute to a proinflammatory environment include a number of cytokines from both T cells and antigen-presenting cells (APCs) (e.g., IL-12, IFN-γ), the strength of activation, and the infectious context. Activated autoreactive T cells adhere to the blood brain barrier (BBB) endothelium via adhesion molecules (LFA-1 and VLA-4), transmigrate into the brain parenchyma through cerebrovascular unit and extravasate into the CNS.

Once in the CNS, the autoreactive $CD4^+$T cells initiate myelin destruction through the activation of resident and infiltrating APCs. The activated infiltrating immune cells secrete cytokines and chemokines that not only recruit immune cells into the central nervous system, but also help to open the BBB. Besides the re-activation of the $CD4^+$ T cells that are specific for the initiating antigen, myelin antigens are released, phagocytosed, processed, and presented principally within the CNS by peripherally derived myeloid dendritic cells (DCs) to naive $CD4^+$ T cells, both of which can enter through the compromised BBB (Fig.4). Several

mechanisms are still unclear, including what guides autoreactive $CD4^+$ T cells to the CNS.

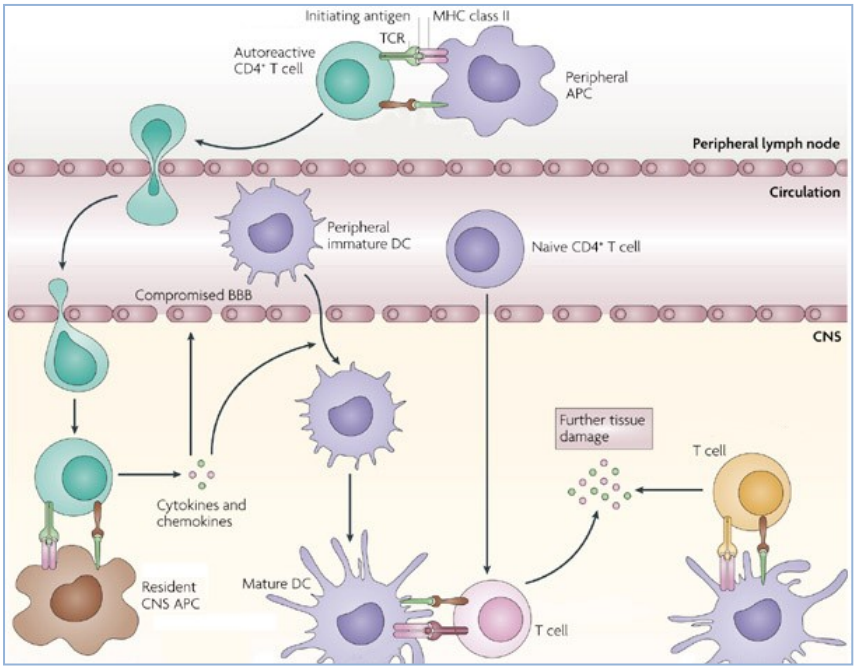
Subsequently, proinflammatory cytokines (IFN-*γ* , IL-23, TNF-*α*, LT) and chemokines (RANTES, IP-10, IL-8, and others) (*a*) activate resident cells, such as microglia and astrocytes; (*b*) recruit other immune cells, including monocytes, $CD8^+$ T cells, B cells, and mast cells, from the peripheral blood; and (c) orchestrate the formation of the inflammatory lesion.

The formation of the inflammatory lesion is characterized by an open BBB with tissue edema after mediator/protease release from mast cells, monocytes, and T cells.

Damage of CNS tissue, i.e., the myelin sheath, oligodendrocytes, and axons, occurs already at this early inflammatory stage. During the above steps, $CD4^+$ autoreactive T cells are likely driving the process, whereas their role in the effector phase is probably secondary. Numerous processes may lead to myelin/oligodendrocyte and axonal damage, including radicals, TNF-α and direct complement deposition, as well as antibody-mediated complement activation and antibody-dependent cellular cytotoxicity via Fc-receptors, myelin phagocytosis, direct lysis of axons by $CD8^+$ cytotoxic T lymphocytes, the secretion of proteases, and apoptosis of oligodendrocytes.

Damage of the target tissue, the central nervous system, is, however, most likely mediated by other components of the immune system, such as antibodies, complement, $CD8^+$ T cells, and factors produced by innate immune cells. Perturbations in immunomodulatory networks that include Th2 cells, regulatory $CD4^+$ T cells, NK cells, and others may in part be responsible for the relapsing-remitting or chronic progressive nature of the disease. However, an important paradigmatic shift in the study of MS has occurred in the past decade. It is now clear that MS is not just a disease of the immune system, but that factors contributed by

the central nervous system are equally important and must be considered (Sospedra and Martin, 2004).



(Adapted from Miller et al., Nature Reviews Immunology, 2007)

Figure 4. Epitope spreading cascade during progression of Multiple Sclerosis. The activation of the autoreactive $CD4^+$ T cells that are specific for the initiating antigen epitope ($CD4^+$ T cell depicted in green) occurs in the draining lymph node. Following activation, the effector $CD4^+$ T cells enter the circulation and extravasate into the CNS. Once in the CNS, the autoreactive $CD4^+$ T cells initiate myelin destruction through the activation of resident and infiltrating antigen-presenting cells (APCs). The activated infiltrating immune cells secrete cytokines and chemokines that not only recruit immune cells into the central nervous system (CNS), but also help to open the blood–brain barrier (BBB). Besides the re-activation of the $CD4^+$ T cells that are specific for the initiating antigen, myelin antigens are released, phagocytosed, processed, and presented principally within the CNS by peripherally derived myeloid dendritic cells (DCs) to naive $CD4^+$ T cells, both of which can enter through the compromised BBB. TCR, T-cell receptor; MHC (major histocompatibility complex) class II

2.7 Glial Cells in Multiple Sclerosis

Glial cells are fundamental for the control of brain homeostasis, (Fig.5) and they represent the intrinsic brain defence system. Neuroglia cells are a major component of pathogenesis of neurological disease. Neuroglia as a major element of neuropathological processes was

already understood by Rudolf Virchow, who was convinced that "*Nervenkitt"/Neuroglia* is central for neuropathology: "*This very interstitial tissue of the brain and spinal marrow is one of the most frequent seats of mordib change*" (Virchow, 1858). All neurological diseases, including MS, can be defined as homeostatic failures. Glia are the principal homeostatic cells of the nervous system and, accordingly, they are integral to homeostatic failures in all neurological diseases. For a long time, our perception of brain pathology has naturally focused on neurons and on their survival or death. This perception is now being challenged, and neuroglia are beginning to be recognized as a central element of neurological disease (Giaume et al., 2007; Verkhatsky et al., 2012). Neurological disorders reflect several levels of compromised tissue homeostasis, and the pathological response of glia determines the degree of homeostatic loss. Multiple Sclerosis can be considered a disease of oligodendrocytes, but all neural cell types are involved (Compston and Coles, 2008). The pathological response of glia largely determines the outcome and scale of neurological disease. The function of neuroglia is neuroprotection in MS, glia attempt to maintain tissue and cellular homeostasis. However, glia can also be neurodestructive, which serves to clean up the compromised tissue and protect the surrounding uncompromised cells/tissue (Verkhratsky and Butt, 2013). The precise mechanisms that determine the progression of MS, and the role of different glial cellular elements in it, remains unresolved.

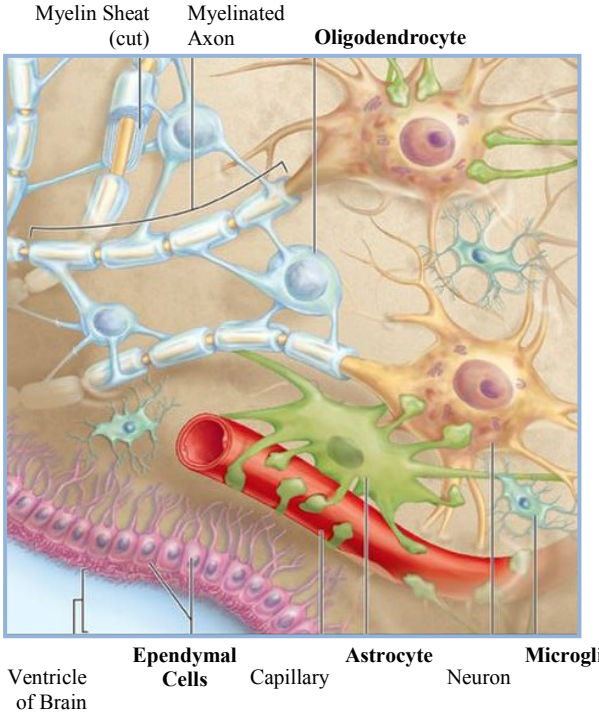


Figure 5. Glia as homeostatic cells of the Nervous System. Neuroglia in the CNS is represented by highly heterogeneous cellular populations of different origin, structure and functions: Ependymal Cells, Astrocytes, Microglia and Oligodendrocytes. One unifying fundamental property common for all these cell types is their ultimate function, the homeostasis of the nervous system. This homeostatic function of neuroglia is executed at many levels, and includes: whole body and organ homeostasis, cellular, morphological, molecular , metabolic, long-range signaling and defensive homeostasis.

2.7.1 Microglia

Microglia play the central role in the immune and inflammatory response in MS, releasing pro- and anti-inflammatory cytokines and chemokines. The accumulation of T and B lymphocytes and the release of pro-inflammatory cytokines recruits naïve microglia, which is key step in the amplification and local spread of the inflammatory response. Activated microglia contribute to inflammation by the release of cytokines, with destructive roles (e.g. TNFα and INFγ), and with roles in repair and remyelination (e.g. IL-10). Microglia are also important in repair by removal of myelin debris, which is inhibitory for remyelinating NG2-glia. Chronic activation of microglia leads to neuronal loss in later stages of the disease (Verkharatsky and Butt, 2013).

2.7.2 Astrocytes

The reactive astrocytes of MS lesions frequently contain myelin or myelin degradation products. In the majority of chronic inactive white matter MS lesions, there is prominent astrogliosis, with a dense meshwork of glial fibrillary acidic protein (GFAP)-positive processes. In active lesions, large hypertrophic astrocytes may be present, which may appear to be in close proximity or even contain the cell bodies of one or more oligodendrocytes. This process seems to be different from phagocytosis.

Astrogliosis is also detected in diffusely abnormal white matter areas with concomitant microglial activation and BBB abnormality. The cause of the general astrocyte activation outside of lesions is unclear, possibly related to inflammatory mediators, diffusing from lesions, stimulating this proliferation. In contrast, in purely cortical lesions, astroglial changes are small or absent.

Astroglial scar has been thought to impair the recruitment of Oligodendrocyte Precursor Cells (OPCs) in MS lesions, although direct evidence is lacking (Lars Bø et al., 2013).

2.7.3 Oligodendrocytes

The pathological hallmarks of MS is the white matter lesion, caused by loss of myelin and oligodendrocytes.

Oligodendrocyte loss has been postulated by Barnett (Barnett and Prineas, 2004) to be the initial event in MS lesion formation. Certainly in the center of chronic inactive lesions, oligodendrocytes are in general not detected, but in other lesion types, there is a large degree of variability of oligodendrocyte numbers, as high numbers of

oligodendrocytes may be present both in active lesions and at the inflammatory edge of chronic active lesions.

A recent study indicates that this variability may be mainly interindividual, as in a subgroup of MS patients a high number of oligodendrocytes were retained in lesion areas (Patrikios et al., 2006). In the chronic MS lesions, oligodendrocyte processes were extended to axons but did not seem to be able to initiate the formation of myelin. This suggests that there are molecules expressed by demyelinating axons in MS lesions that inhibit the initiation of myelination.

The extent of oligodendrocyte loss in gray matter lesions is similar to that in white matter lesions. The mechanisms of oligodendrocyte death in MS are not well characterized (Lars Bø et al., 2013).

2.7.4 Oligodendrocyte Precursor Cells (OPCs)

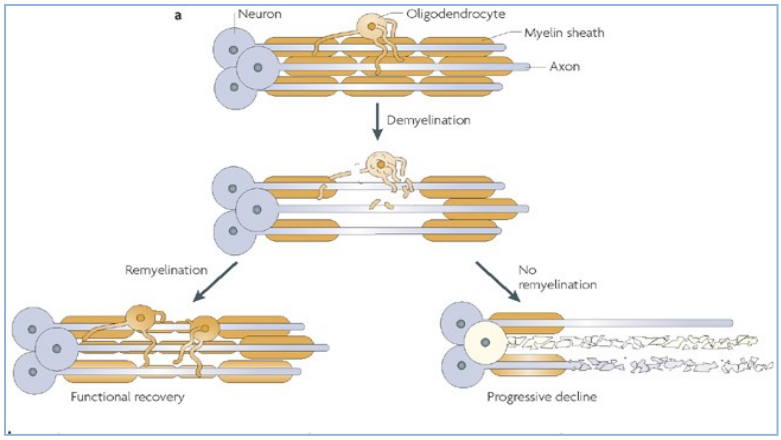
In MS, oligodendroglial precursor cells (OPCs) are believed to be the cells responsible for remyelination.

“Remyelination” or “myelin repair” is the process in which entire myelin sheaths are restored to demyelinated axons, reinstating saltatory conduction and resolving functional deficits.

Following demyelination in the CNS, a demyelinated axon has two possible fates (Fig.6). The normal response to demyelination is spontaneous remyelination involving the generation of new oligodendrocytes. In some circumstances, however, and notably in MS, remyelination fails, leaving the axons and even the entire neuron vulnerable to degeneration that largely accounts for the progressive clinical decline (Franklin and ffrench-Constant, 2008).

In demyelinated lesions depleted of oligodendroglial lineage cells, OPCs occur with onset of remyelination suggesting that OPCs and not mature oligodendrocytes are responsible for remyelination (Fancy et al.,

2004; Levine and Reynolds, 1999; Watanabe et al., 2002). In the adult brain and spinal cord, OPCs, also known as NG2 cells, are ubiquitous and represent a large percentage of the total cell population, as much as 9% of cells in white matter and 3% in gray matter (Nishiyama, 2001). In response to a demyelinating lesion, NG2 cells start proliferating and are recruited to the demyelinated axons, where they differentiate into mature, myelin-forming oligodendrocytes. In response to injury, local OPCs undergo a switch from an essentially quiescent state to a regenerative phenotype. This activation is the first step in the remyelination.



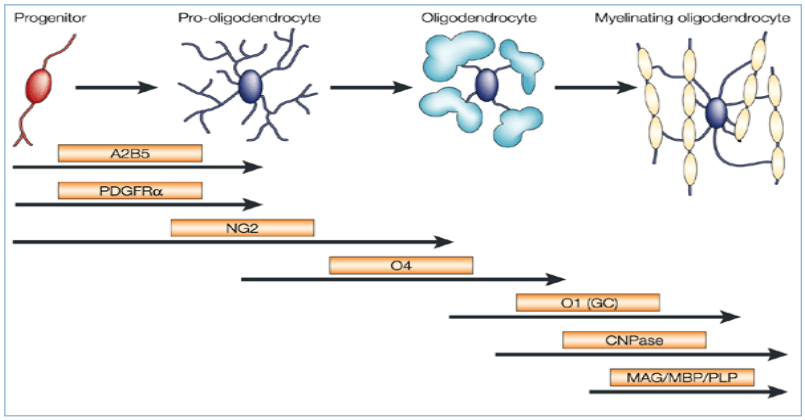
Adapted from Franklin and ffrench-Constant., Nature Neuroscience, 2008

Figure 6. The fate of demyelinated axons. a. Following demyelination in the CNS, a demyelinated axon has two possible fates. The normal response to demyelination, at least in most experimental models, is spontaneous remyelination involving the generation of new oligodendrocytes. In some circumstances, however, and notably in Multiple Sclerosis, remyelination fails, leaving the axons and even the entire neuron vulnerable to degeneration that largely accounts for the progressive clinical decline that is associated with demyelinating diseases.

Following recruitment, the OPCs must differentiate into remyelinating oligodendrocytes. This differentiation phase encompasses three distinct steps: establishing contact with the axon that is to be remyelinated, expressing myelin genes and generating a myelin membrane, and finally wrapping and compacting the membrane to form the sheath.

OPCs proliferate and migrate before differentiating into late oligodendrocyte progenitors [premyelinating oligodendrocytes (preOLs)]

which undergo extensive cellular growth and process elaboration and mature into myelinating oligodendrocytes (OLs) (Jakovcevski and Zecevic, 2005; Kessaris et al., 2006; Jakovcevski et al., 2009; Rowitch, 2004). The different stages of maturation toward myelinating oligodendrocytes can be identified by cell type-specific markers. In the past decade, various markers have been identified that show lineage- and stage-specific expression. The markers platelet-derived growth factor receptor-alpha (PDGFRα), NG2, a membrane chondroitin sulfate proteoglycan, Olig2, Nkx2.2, Sox10, and Olig1 (nuclear) are characteristic of OPCs. O1 [also known as galactocebroside (GalC)], O4 antigen and 2'-3'-cyclic nucleotide-3'-phosphodiesterase (CNPase) mark intermediate preOLs, whereas myelin basic protein (MBP), myelin proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG) and Olig1 (cytoplasmic) are typical of mature myelinating oligodendrocytes. (Fig.7)



Adapted by Zhang, Nature Neuroscience, 2001

Figure 7. Morphological and antigenic markers for oligodendroglia during development. The differentiation of oligodendrocytes from their progenitors follows a stepwise morphological transformation from bipolar progenitors to pro-oligodendrocytes bearing multiple processes (immature), membrane-sheath-bearing mature oligodendrocytes, and, finally, myelinating oligodendrocytes. Accompanying this morphological change is the sequential expression of molecular markers: A2B5 antigen, platelet-derived growth factor receptor-(PDGFR) and chondroitin sulphate proteoglycan NG2 in progenitors, O4 antigen in pro-oligodendrocytes, galactocerebroside (GC or GalC or O1 antigen) and 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) in mature oligodendrocytes, and most myelin proteins - myelin-associated glycoprotein (MAG), myelin basic protein (MBP) and proteolipid protein (PLP) - in myelinating oligodendrocytes.

2.8 Current treatments and future perspectives in therapeutic strategies for Multiple Sclerosis

Currently, the drugs for MS are exclusively immunomodulatory therapies. These drugs are relatively effective at preventing new demyelinated lesions from forming and significantly impeding relapsing-remitting disease progression, but are ineffective at preventing the transition to or advancement of progressive MS. At this chronic stage neurodegeneration becomes increasingly evident leading to the accumulation of irreversible clinical disability (Compston and Coles, 2008). At present, approximately 50% of people affected by MS are at the progressive stage of the disease illustrating the critical need for developing additional therapeutic strategies to protect oligodendrocytes and bolster regeneration in combination with current immunotherapies.

There are three principal approaches to treating MS: 1) halting the pathologic immune response, 2) protecting the CNS from further damage, and 3) repairing the damage through the regeneration of new myelin sheaths, with the overarching goals being to restore conduction and prevent further axonal loss (Rodgers et al., 2013).

The landscape for MS therapies is undergoing a rapid expansion as knowledge accumulates on how aberrant immune responses produce demyelinating injury and how the CNS is able to regenerate myelin. The early broad-based immunosuppressive drugs are giving way to targeted, antigen-specific approaches minimizing side effects and maximizing clinical benefit. At the same time decades of work suggest that immunomodulation alone will only go so far and that strategies to protect oligodendrocytes and promote remyelination should be considered in parallel, combinatorial therapeutic approaches. Many repurposed drugs are showing promise for protecting oligodendrocytes, and remyelination strategies focusing on antibodies and

pharmacological targets have shifted the focus from cellular replacement to enhancing endogenous repair (Rodgers et al., 2013).

2.8.1 Immune-modulating Therapies

The ten existing disease-modifying treatments for MS target the immune compartment (Derwenskus, 2011). Early approaches aimed to limit activation of pathologic immune cells were relatively non specific in their scope. Early drugs interferon-beta (Avonex, Rebif, Betaseron Extavia) and glatiramer acetate (Copaxone), a synthetic copolymer, are both administered subcutaneously to suppress multiple cell types including antigen presenting cells and TH1 and TH17 helper subsets cells and shift the immune system towards a regulatory phenotype (Lalive et al., 2011). Mitoxantrone (Novatrone), a synthetic antineoplastic drug, induces apoptosis in highly proliferative cells and suppresses macrophages, B cells, and TH cells (Derwenskus, 2011). Recent work in autoimmunity has sought to refine the therapeutic approach in new ways, more specifically targeting the pathologic immune compartment without compromising the entire arm of the adaptive immune response thus minimizing side effects and any risks of opportunistic infection. Two current therapies limit T cell migration: fingolimod (Gilenva) constrains T cell migration from lymph nodes, whereas natalizumab (Tysabri) blocks T cell infiltration across the blood-brain barrier into the CNS.

2.8.2 Oligodendrocyte Protective Therapies

The majority of drugs under investigation to protect oligodendrocytes from apoptosis were first discovered for their immunomodulatory or neuroprotective capabilities in other capacities. In the context of MS, it

is often poorly understood whether the beneficial effects are due to immune modulation, neuroprotection, or both. Indeed uncoupling these effects is particularly difficult as CNS degeneration is so intimately associated with inflammation. In light of a combination therapeutic approach, drugs that protect oligodendrocytes from apoptosis are likely to be most effective when used in combination with systemic immune modulation whether in cases of acute or chronic inflammation. Current data highlight the importance of proper timing in CNS-protective drug treatments as some prove to be most efficacious in early relapsing-remitting disease associated with deleterious inflammation. In this vein neuroprotective drugs may be unnecessary once immune tolerance has been established, particularly if tolerance can be induced early in disease and proves to have long-lasting effects (Rodgers et al., 2013).

2.8.3 Remyelination Enhancing Therapies

The third component to a combinatorial therapeutic approach for MS is to enhance regeneration in the absence of the pathologic immune response and primary CNS damage. Whilst there are no remyelination-enhancing therapies clinically available at present, research in this area is developing, with the focus on two major approaches: transplantation of exogenous cells and stimulation of the endogenous repair process. Both approaches have their advantages and disadvantages, and it has become clear, particularly in recent studies, that is not possible to draw a complete distinction between these two approaches (Crawford et al., 2013).

2.9 Animal Models in Multiple Sclerosis Research

A number of animal models have been developed in order to understand a variety of aspects of human MS. The main driving force for animal studies stems from the following limitations of human studies: overall limited access to human MS tissue, biopsies are rarely performed and autopsy samples are usually biased towards a chronic, burnt-out stage; experimental circumstances can't easily be modified in clinical trials and mechanistic studies addressing disease pathomechanisms can't readily be performed in patients.

There is no single animal model that can capture the entire spectrum of heterogeneity of human MS and its variety in clinical and radiological presentation. However, over the last several decades, useful and relevant animal models have been developed that represent selected aspects of the human disease. Depending on the specific research question, the rational selection of appropriate animal models is likely to yield outcomes that will result in translatable findings applicable to MS. Despite the clearly existing limitations, basic science MS research will continue to rely on these models for new drug development and for understanding the ramifications and diversity of pathomechanism in MS (Denic A. et al., 2011).

The most commonly studied animal models of MS are (1) the purely autoimmune experimental autoimmune/allergic encephalomyelitis (EAE), (2) viral induced models, mainly Theiler's murine encephalomyelitis virus (TMEV) infection and consequential chronic demyelination and (3) toxin-induced models of demyelination, including the cuprizone model and focal demyelination induced by lyso-phosphatidylcholine (lysolecithin).

2.9.1 Experimental Autoimmune Encephalomyelitis (EAE)

Experimental Autoimmune Encephalomyelitis (EAE) is the most commonly used experimental model for the human disease MS. EAE is a complex condition in which the interaction between a variety of immunopathological and neuropathological mechanisms leads to an approximation of the key pathological features of MS: inflammation, demyelination, axonal loss and gliosis. The counter-regulatory mechanisms of resolution of inflammation and remyelination also occur in EAE, which, therefore can also serve as a model for these processes. EAE is a good model for studying MS mechanisms, even more so than for testing or developing drugs (Farooqi et al., 2010).

A major difference between MS and EAE is that the latter requires an external immunization step to develop, whereas in humans, the sensitization to autoantigens is obviously not artificially induced (Gran et al., 2007). Sensitization to myelin antigens in EAE typically occurs through the use of adjuvant, usually containing bacterial components highly capable of activating the innate immune system via pattern recognition receptors (Libbey and Fujinami, 2010).

EAE can be induced in a multitude of species and strains. Interestingly, humans were the first species where sensitization with nervous tissue led to an inflammatory demyelinating CNS disease. This occurred as a rare complication of rabies vaccination with virus grown on rabbit spinal cord (Sabin and Wright, 1934). It was subsequently shown that the resultant encephalomyelitis was not due to rabies, but to an autoimmune response triggered by the spinal cord contaminant of the vaccine. Rivers in 1933 developed EAE in an attempt to understand better the pathogenesis of this post-vaccinal encephalomyelitis (Zinsser and Tang, 1926; Rivers and Stewart, 1928; Rivers et al., 1933; Rivers and Schwentker, 1935). Since then, EAE has been induced in a variety

of rodents and monkeys, providing models of acute monophasic, relapsing–remitting and chronic progressive CNS inflammation.

'Active' EAE is induced by immunization with CNS tissue or myelin peptides, such as MBP, MOG and PLP in Complete Freund's Adjuvant (CFA), with high incidence of disease induced in susceptible animal strains (Stromnes and Goverman, 2006).

By contrast, 'passive' or adoptive-transfer EAE (AT-EAE) can be induced in recipient animals by transferring pathogenic, myelin-specific $CD4^+$ T cells generated in donor animals by active immunization (Stromnes and Goverman, 2006). The latter type of EAE was instrumental in establishing the key role of myelin-reactive T cells in disease pathogenesis (Pettinelli and McFarlin, 1981).

A frequently used EAE model is induced in the C57BL/6 mouse by immunization with $MOG_{35–55}$ in CFA. The immunization can induce monophasic or a chronic, sustained form of EAE. The former is characterized by multifocal, confluent areas of mononuclear inflammatory infiltration and demyelination in the peripheral white matter of the spinal cord (Day, 2005). Macrophages and $CD4^+$ T cells are the main cell types in the inflammatory infiltrate. In the brain, there is meningitis and perivascular inflammatory cuffing in the cerebellum and hindbrain white matter.

In conclusion, EAE has contributed to the development, validation, and testing of MS drugs and even more remarkably, to the understanding of the pathogenesis of MS.

2.9.2 Virus Induced Demyelination

Epidemiological studies have put forward a hypothesis that a viral infection early in life, in the presence of a specific genetic background, may result in immune mediated attack against CNS tissue (Poser,

1986). However, to date there is no specific virus that has been identified as a potential cause or contributor to MS (McFarlin and Mc Farland, 1982). To model the contribution of viruses in human MS, some investigators utilize virus induced demyelination models. Experimentally, demyelinating disease in susceptible mouse strains is induced by intracerebral infection with TMEV. TMEV (Theiler's murine encephalomyelitis virus) is a mouse enteric pathogen that belongs to the single-stranded RNA picornaviruses. Unlike EAE, the disease is always chronic-progressive in susceptible mice and there is no consensus about the exact mechanism of demyelination in the TMEV model (Denic A. et al., 2011).

2.9.3 Toxin Induced Demyelination

The two most widely used toxins to induce demyelination in animal models of MS are lysolecithin and cuprizone. Lysolecithin is an activator of phospholipase A_2 and cuprizone is a copper chelator. Compared to EAE and virus induced demyelinating syndrome, toxin induced demyelination models do not attempt to mimic MS as a disease, but are mainly established as systems to study the process of focal demyelination and remyelination (Blakemore and Franklin, 2008).

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II. AIM OF THE STUDY

MS is an inflammatory demyelinating autoimmune disorder of the CNS. Current medical treatment mainly influences disease progression via immunomodulatory or immunosuppressive actions. Indeed, MS research has been foremost focused on inflammation in the CNS, but more recent evidence suggests that chronic disability in MS is caused by neurodegeneration (Ellwardt and Zipp, 2014). Basic research on the MS has been performed mainly on its animal model namely Experimental Autoimmune Encephalomyelitis (Trafreshi et al., 2005). EAE is a complex condition in which the interaction between a variety of immunopathological and neuropathological mechanisms leads to an approximation of the key pathological features of MS: inflammation, demyelination, axonal loss and gliosis. The counter-regulatory mechanisms of resolution of inflammation and remyelination also occur in EAE, which, therefore can also serve as a model for these processes (Constantinescu et al., 2011).

The dysregulation of $[Ca^{2+}]_i$ and $[Na^+]_i$ homeostasis is involved in neuronal and glial injury occurring in several neurodegenerative diseases, including MS. The Na^+/Ca^{2+} exchanger (NCX), a transmembrane domain protein, which, by operating in a bidirectional way, couples the efflux of Ca^{2+} to the influx of Na^+ into the cells or, viceversa, the influx of Ca^{2+} to the efflux of Na^+, is involved in the regulation of diverse neuronal and glial cell functions (Annunziato et al., 2004). Recent evidence point out that the isoform-3 of the Na^+/Ca^{2+} exchanger is a new potential therapeutic target for neuroprotection (Pignataro et al., 2004; Molinaro et al., 2008; Pignataro et al., 2011). In agreement with the beneficial role of this exchanger, it has been demonstrated that NCX3-deficient mice showed skeletal muscle fiber necrosis and impaired neuromuscular transmission, which is clinically

associated with reduced motor activity, weakness of forelimb muscles, and fatigability (Sokolow et al., 2004).

In a paper recently published by our research group it has been demonstrated that calcium signaling mediated by NCX3 isoform has a crucial role in the progression of OPCs into mature oligodendrocytes. Interestingly, spinal cords isolated from ncx3-/- mice were smaller than those isolated from wild-type, congenic, ncx3+/+ mice. Furthermore, NCX3-deficient mice showed hypomyelination that is accompanied by an augmented expression of the OPC marker NG2 and a reduction of spinal cord size (Boscia et al., 2012). More recently, in an attempt to understand the molecular composition of myelin membranes, Gopalakrishnan et al., (2012) identified the isoform-3 of Na^+/Ca^{2+} as a common protein to human and murine myelin proteome. Therefore, the decreased myelination observed in the ncx3-/- mice (Boscia et al., 2012) might be caused by a decrease in the number of differentiated oligodendrocyte and/or by a decreased ability of oligodendrocytes to produce myelin.

In light of this, in the present study we asked whether the NCX3 exchanger might have a role in the susceptibility of the disease and during the pathophysiological responses in a mouse model of Multiple Sclerosis. To address these questions we used the EAE as a murine model for MS.

The study objectives included:

1) To assess all inflammatory, neuronal and glial responses after EAE immunization. Morphological analyses were performed at different time points during disease progression in order to characterize the patterns of demyelination, axonal loss and glial activation.

2) To provide a clear picture of NCX3 expression and distribution in the white matter spinal cord during EAE course. Biochemical and immunohystochemical analyses were performed with the aim to analyze the expression profile of NCX3 protein in the spinal cords of control and EAE mice. Co-expression analyses of NCX3 with both neuronal and oligodendrocyte lineage markers were performed in order to evaluate the involvement of this isoform during EAE responses.

3) To establish the role played by NCX3 in EAE severity and progression. Behavioural and morphological analyses were performed in NCX3 congenic wild type and in NCX3 knockout mice exposed to EAE with the aim to study the neurological deficits, the demyelination, the axonal loss and the oligodendroglial responses during the pathology.

III. MATERIALS AND METHODS

1. Animals

Experiments were performed on C57BL/6, ncx3 congenic wild-type (ncx3+/+), ncx3 heterozygous (ncx3+/-) and knockout (ncx3-/-) mice (Sokolow et al., 2004). C57BL/6 mice were purchased by Charles River. The generation of NCX3 knockout mouse has been described by Sokolow et al. (2004). In particular, the ncx3 gene includes nine exons spanning a region of about 150 kb. To constructed a mutant allele, the exon 2 of the murine ncx3 gene (GeneBank, accession number AF321404), which encodes for the first two-thirds of the protein, was cloned from a 129/Sv genomic phage library. A targeting vector was constructed by inserting the neomycin resistance cassette (neo) into the unique EcoRI restriction site of that exon. The insertion of the neomycin resistance cassette in the second exon of the ncx3 gene created a null mutation. After electroporation with the targeting vector, the recombinant embryonic stem (ES) clones were identified by DNA hybridization and used to produce chimeric mice. Transmission of the mutant allele produced NCX3 heterozygous mice (ncx3+/-) that were mated to generate NCX3 null mutants (ncx3-/-) (Sokolow et al., 2004). Female mice, aged between 6-8 weeks and weighing 19-20 g were housed under diurnal lighting conditions (12 hr darkness and 12 hr light) and had free access to food and water. All of the mouse models were bred in ventilated rooms, in a germ-free facility, under controlled conditions of light, temperature and humidity. Experiments were performed on mice according to the international guidelines for animal research and approved by the Animal Care Committee of “Federico II” University of Naples, Italy.

2. Genotyping of Mice

DNA extraction from mouse tail. To genotype the mice, DNA was extracted from mouse tail. From 0.7 to 1 cm of the mouse tail was incubated in 600 µl digestion buffer containing proteinase K (1 mg/ml) at 55°C over night. At the digestion mixture were added 500µl of Phenol:Chloroform:Isoamyl Alcohol (25:24:1) and it was centrifuged at 13.000 rpm for 10 minutes at room temperature to collect undigested tail debris. Finally, the DNA containing supernatant was removed and was added 1 ml of cold 95% Ethanol and inverted to mix. The DNA was isolated and resunspended in 50 µl of steril water and was allowed to dissolve.

Standard Polymerase Chain Reaction (PCR) Amplification. Genotyping was performed using PCR amplification. PCR was carried out in 200µl thin walled PCR tubes using 1 µl of DNA, 2 µl of 2.5 mM of dNTPs consisting of dATP, dTTP, dGTP and dCTP (Lucigen), 0.125 µl of Taq polymerase (EconoTaq DNA Polymerase 1000 U at 5U/µl, Lucigen), 2.5 µl of 10x Buffer (EconoTaq Buffer with Mg, Lucigen),1 µl of 0.2 µg/µl forward primer, 1 µl of 0.2 µg/µl reverse primer and 17.37 µl of sterile filtered distilled water. In one reaction, two primer pairs were used to amplify regions of the target genes. Positive and negative control samples were also used. A positive control (DNA of known genotype) was used to make sure all the reagents were still working appropriately. In addition to this, a negative control containing sterile filtered water was included to make sure the reaction mixture was not contaminated with another source of DNA.

Agarose Gel Electrophoresis. PCR products were separated by electrophoresis on a 1.5 % agarose gel, and visualised under UV light followed ethidium bromide staining. A 100 kb DNA ladder was also loaded onto the gel to permit size analysis of PCR products.

Electrophoresis was performed at 100 v for approximately 45 minutes allowing the fragments to completely separate. The DNA bands were visualised by placing the gel into a UV transluminator.

3. Active Induction of EAE in Mice

EAE was induced by active immunization with the peptide corresponding to the immunodominant epitope of Myelin Oligodendrocyte Glicoprotein (MOG), the peptide 35-55 (p35-55). A p35-55 sequence of MOG (MOG_{35-55}): M-E-V-G-W-Y-R-S-P-F-S-R-V-V-H-L-Y-R-N-G-K was synthesized by INBIOS srl, Naples, Italy. Mice were immunized with 200µg of MOG_{35-55} emulsified in Complete Freund's Adjuvant (CFA) (DIFCO) supplemented with 4 mg/ml killed mycobacterium tuberculosis. A volume of 0.1 ml of the emulsion was injected subcutaneously (s.c.) for each mouse. Mice also were administered intraperitoneal (i.p.) pertussis toxin (PT) (Sigma). PT was injected immediately and 48 hours later. Pertussis toxin can promote the activation of T cells and contribute to the destruction of blood-brain barrier (Hofstetter et al., 2002). PT enhances EAE development by providing additional adjuvant and facilitating entrance of autoimmune T cells into the CNS. Control mice were injected with equivalent amounts of CFA alone.

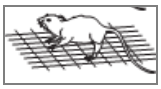
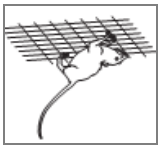
4. Clinical Scoring of EAE

Mice were monitored daily, starting on the day of the immunization (day 0) and continuing until day 50. MOG-immunized mice were checked for clinical signs of EAE and graded on a scale of increasing severity. Clinical signs and ascending paralysis were assessed on a six-stage scale of 0-5. Disease scores were assigned as shown in Table 1.

Fractional scores result either from calculating the mean of scores from a group of animals or from assigning fractional scores for partial but distinct phenotypes; for example, when the distal tail droops while the tail can be raised overall, a score of 0.5 was assigned and when one leg is dragging but the other bears a large fraction of the body's weight, a score of 2.5 or 2.75 was assigned.

Table 1. Clinical Scoring of EAE

Score	Clinical Observations
0.0 	No obvious changes in motor function compared to non-immunized mice. When picked up by base of tail, the tail has tension and is erect. Hind legs are usually spread apart. When the mouse is walking, there is no gait or head tilting.
0.5	Tip of tail is limp. When picked up by base of tail, the tail has tension except for the tip. Muscle straining is felt in the tail, while the tail continues to move.
1.0 	Limp tail. When picked up by base of tail, instead of being erect, the whole tail drapes over finger. Hind legs are usually spread apart. No signs of tail movement are observed.
1.5	Limp tail and hind leg inhibition. When picked up by base of tail, the whole tail drapes over finger. When the mouse is dropped on a wire rack, at least

	one hind leg falls through consistently. Walking is very slightly wobbly.
2.0 	Limp tail and weakness of hind legs. When picked up by base of tail, the legs are not spread apart, but held closer together. When the mouse is observed walking, it has a clearly apparent wobbly walk. One foot may have toes dragging, but the other leg has no apparent inhibitions of movement.
2.5	Limp tail and dragging of hind legs. Both hind legs have some movement, but both are dragging at the feet (mouse trips on hind feet). - OR - No movement in one leg/completely dragging one leg, but movement in the other leg.
3.0 	Limp tail and almost complete paralysis of hind legs. One or both hind legs are able to paddle, but neither hind leg is able to move forward of the hind hip. - OR - Limp tail with paralysis of one front and one hind leg.
3.5	Mouse is moving around the cage, but the hind quarters are flat.
4.0 	Limp tail, complete hind leg and partial front leg paralysis. Mouse is minimally moving around the cage but appears alert and feeding.

4.5	Complete hind and partial front leg paralysis, no movement around the cage. Mouse is not alert. Mouse has minimal movement in the front legs. The mouse barely responds to contact. Euthanasia is recommended. When the mouse is euthanized because of severe paralysis, a score of 5.0 is entered for that mouse for the rest of the experiment.
5.0 	Mouse is spontaneously rolling in the cage (euthanasia is recommended). - OR - Mouse is found dead due to paralysis. - OR - Mouse is euthanized due to severe paralysis.

5. Western Blotting

Spinal cord tissues were collected for biochemical studies at different time points after immunization. Protein samples, isolated using tissue lysis buffer, were separated on 14% polyacrylamide gel and electrophoretically transferred onto nitrocellulose membranes. The membranes were blocked with 5% non fat dry milk. Filters were probed using the primary monoclonal antibody anti-NCX3 (1:1000, Trans Genic Inc.) for over night incubation at 4°C. The membranes were incubates with peroxidase-conjuigated secondary antibody for 1 hour at room temperature and proteins were visualized using the enhanced chemiluminescence system (Amersham-Pharmacia Biosciences LTD, Uppsala, Sweden).

6. Immunohistochemistry (IHC)

Mice were anesthetized with gas anesthesia (SevoFlo, Abbott) and perfused transcardially with 4% wt/vol. paraformaldehyde and 15% wt/vol. picric acid in phosphate buffer. Using a fine forceps, vertebra were removed and spinal cords were isolated. Spinal cords were cryoprotected in sucrose, frozen in OCT, and sectioned coronally at 40 μm on a cryostate. The sections were blocked with 3% Bovine Serum Albumin (BSA). After blocking, sections were incubated with primary antibodies for 48 hr. The primary antibodies used in these experiments were the following: polyclonal anti-NF200 (1:1000, Sigma); monoclonal anti-MBP (1:400, Covance); monoclonal anti-Iba1(1:2000, WAKO); polyclonal anti-GFAP (1:1000, Sigma); monoclonal anti-NCX3 (1:300, Trans Genic Inc.); polyclonal anti-MBP (1:2000, Millipore); polyclonal anti-NG2 (1:1000, Millipore); polyclonal anti-GalC (1:500, ProteinTech); polyclonal anti-CNPase (1:200, santa Cruz Biotechnology Inc.). For double immunofluorescence sections were incubated with corresponding fluorescence labeled secondary antibodies (Alexa-488- or Alexa-594- conjugated anti-mouse or anti-rabbit IgGs). Hoechst 33258 was used as nuclear stain. For immunoenzymatic staining, sections were instead incubated with biotinilated secondary antibodies (anti-mouse or anti-rabbit, Vector), the peroxidase reaction was developed using 3-3'-diaminobenzidine/4HCl as a chromogen. Because no immunoreactivity differences were found between control and CFA-injected animals, the data are presented as a comparison of MOG_{35-55}-immunized and CFA-injected mice.

7. Confocal Microscopy and Image Analysis

Images were observed using a Zeiss LSM 710 laser scanning confocal microscope. Single images were taken with an optical thickness of 0.7

mm and a resolution of 1024x1024. All images were obtained with a 20x, 40x, 60x or 100x objectives with identical laser power setting. The fluorescence intensity and the % of colocalized points on coronal tissue sections from spinal cord were quantified in terms of pixel intensity value by using the NIH image software.

The number of cells was determined in the white matter of the spinal cord by manual counting at 40x or 60x magnification only cells with clearly a visible cell body and profiles were counted.

8. Statistical Analysis

The data are expressed as the mean±S.E.M. of the values obtained in three separate experiments. Statistical comparisons between controls and treated groups were performed by one-way analysis of variance followed by Newman–Keuls' test. $P^{*}<0.05$ was considered significant.

IV. RESULTS

1. MOG_{35-55}-induced EAE in C57BL/6 mice reproduced the key pathological features of MS

The EAE clinical course was first characterized by a pre-onset stage (0-10 days post immunization, dpi) then, by an acute disease onset (10-15 dpi) characterized by a peak disease stage (15-25 dpi) followed by a consecutive chronic phase lasting up to 50 days, the latter time point at which the animals were analyzed (Fig.8A). During the pre-onset phase, mice did not show any visible motor defects. The first clinical sign of EAE, indicated by a loss of tail tonicity, was observed around 10 days after the immunization. The increase in clinical score corresponded to the decline in body weight (Fig. 8B). At this time point mice developed a weak or flaccid tail and started to show signs of motor dysfunction. During the peak stage mice progressively lost the ability to move the hind legs and a significant weight loss occurred. At this stage the animals reached the highest clinical EAE score including loss of tail tonicity and paralysis of hindlimbs. The chronic phase was the recovery stage of EAE; most of the mice had a tail that was no longer limp but was not normal either; it felt rigid and was "hooked". The hind legs started moving. During the chronic phase the EAE clinical scores improved. Control mice did not show weight loss or neurological deficits at any time point following CFA injection (Fig. 8A). In order to correlate the clinical motor deficits observed in MOG-immunized mice to the cellular and molecular events occurring during the EAE disease course, we analyzed by means of confocal double immunofluorescence experiments, inflammation, demyelination and axonal loss occurring in the brain and spinal cord of EAE mice. The experiments performed in the cerebellum and in the other brain regions revealed that the lumbo-thoracic spinal cord was much more affected by the EAE model (data

not shown). Thus, we focused our subsequent studies only within this CNS area.

Confocal double immunofluorescence experiments performed with the axonal marker NF200 and the myelin marker MBP in the presence of the nuclear marker Hoechst revealed that a significant reduction in both MBP and NF200 immunoreactivity was detected in several plaques troughout the white matter tracts at peak stage. At this stage, the plaques were easily identified by the intense labelling of cellular infiltrates with the nuclear marker Hoechst (Fig. 8C, c-d). During the chronic stage, the number of infiltrates, detected by Hoechst staining, appeared strongly reduced, and several larger regions of reduced MBP and NF200 fluorescence staining were observed (Fig. 8C, e-f). In order to characterize the pattern of microglial and astocytic activation in EAE spinal cord we performed colocalization experiments with the microglial anti-Iba1 marker and the astrocytic marker anti-GFAP in the presence of the nuclear marker Hoechst. Immunofluorescence experiments revealed that the activation of both microglia and astrocytes was higher 21 dpi both in the white and grey matter of spinal cord (Fig. 8C, i-j). By contrast, their labelling appeared strongly reduced in the grey matter at chronic stage, but it remained still elevated within demyelinated regions at chronic stage (Fig. 8D, k-l).

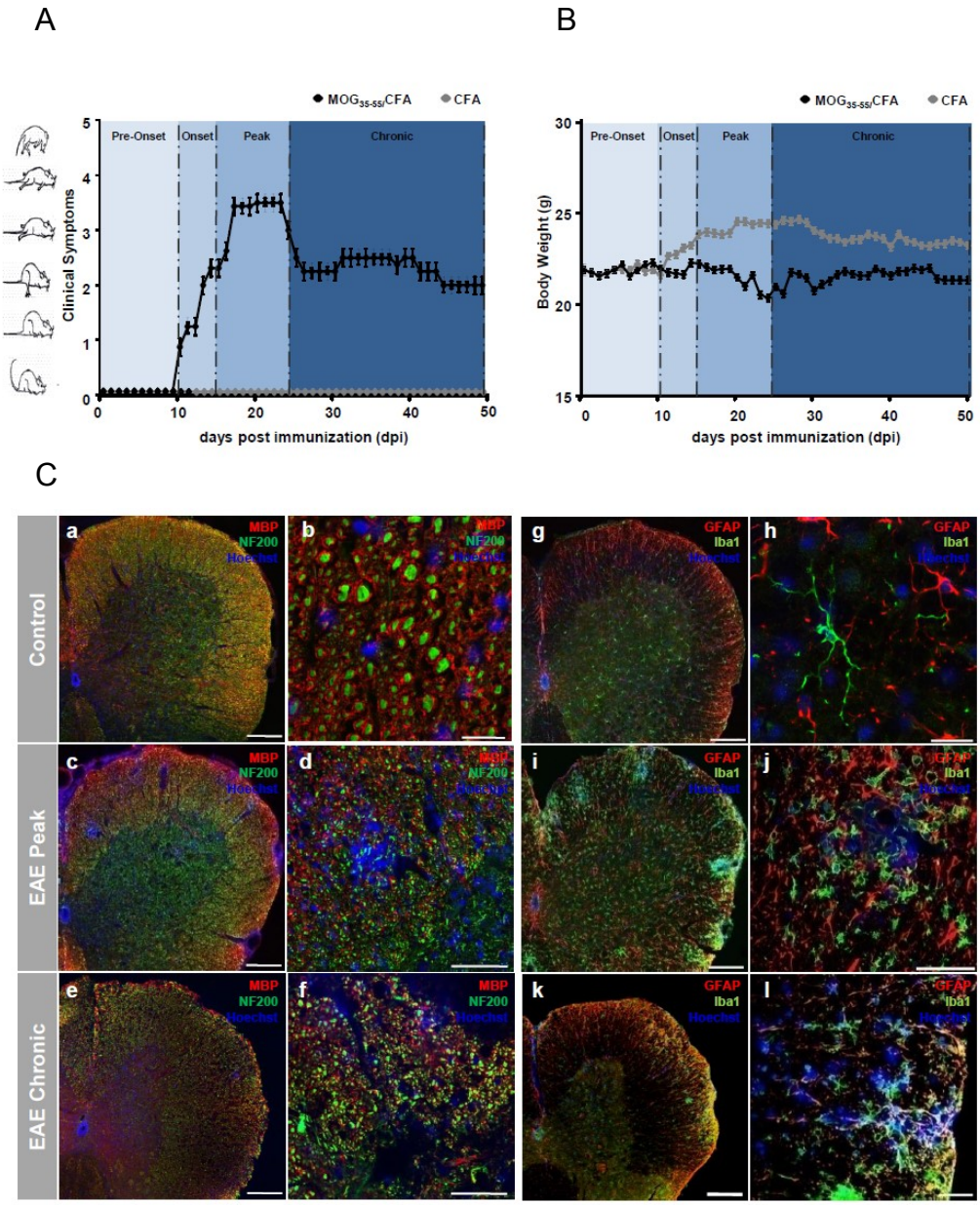


Figure 8. Pathological features of MOG_{35-55}-induced EAE in C57BL/6 mice. Assessment of EAE clinical symptoms **(A)** and body weight progression **(B)** in MOG_{35-55}-immunized mice compared with vehicle-treated mice during pre-onset, onset, peak and chronic stages. Results represent average (mean±S.E.M.) of the data obtained from 6 animals per group in three independent experimental sessions. **(C)** Confocal double-labelling experiments showing MBP (red) - NF200 (green) (a-f) and GFAP (red) - Iba1 (green) (g-l) immunoreactivities in the spinal cord of control (a,b,g,h) and EAE-affected mice during the Peak (c,d,i,j) and Chronic (e,f,k,l) stages. Scale bars: 200µm (a, c, e, g, i, k); 20µm (b,h); 50µm (d,f,j,l).

2. Up-regulation of NCX3 protein levels in the spinal cord during EAE progression

In order to investigate NCX3 protein expression and distribution in spinal cord tissue of healthy and EAE mice, biochemical and immunohistochemical analyses were performed at different time points during EAE disease course.

Western Blotting analysis performed on spinal cord tissue homogenates revealed that NCX3 protein levels were progressively up-regulated during EAE progression. This effect was more significant at chronic stage (Fig. 9A).

Single immunohistochemical experiments performed with anti-NCX3 antibody in the white matter spinal cord of control and EAE mice revealed a very distinctive expression pattern of NCX3 exchanger being localized not only to selective white matter axonal tracts, but also to intensely stained cells which resembled oligodendroglial lineage morphology (Fig. 9B). Among the different axonal tracts, NCX3 exchanger was intensely detected in the lateral tracts (Fig. 9B, a,b,e), moderately detected in the anterior tracts (Fig. 9B, h,i), but barely detected in the posterior tracts (Fig. 9B, l,m). Along the lateral and anterior tracts, the ascendent spinocerebellar tract and the descendent anterocorticospinal tract, appeared the most intensely immunostained. During the peak and chronic stages NCX3 axonal immunoreactivity appeared reduced. At these stages NCX3 staining was detected in a large number of cells with oligodendroglia-like morphology. This effect was particularly evident at chronic stage in the white matter lateral tracts (Fig. 9C, c,d,f,g,j,k,n,o).

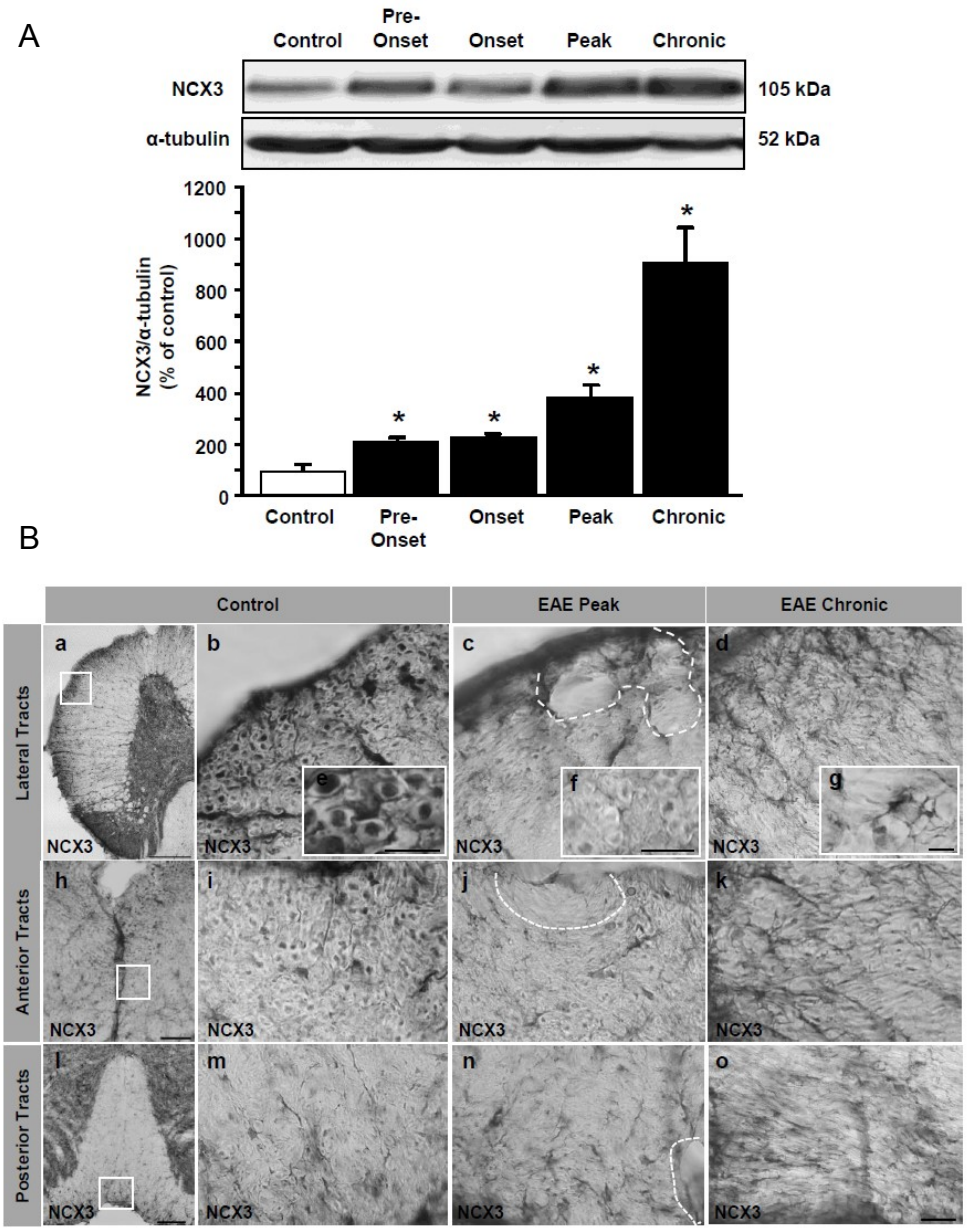


Figure 9. Expression profile of NCX3 protein in the spinal cord of control and EAE mice. (A) Western blot and densitometric analysis of NCX3 protein levels in spinal cord tissue homogenates during EAE progression. The data were normalized on the basis of α-tubulin levels and expressed as percentage of control. The values represent the means±S.E.M. (n=3-4). **P<0.05 versus* control. **(B)** Single immunohistochemical experiments with anti-NCX3 antibody (a-o) in control (a,b,e,h,i,l,m) and in EAE affected-spinal cord during the Peak (c,f,j,n) and Chronic (d,g,k,o) stages. Analysis were performed in the lateral (a-g), anterior (h-k) and posterior (l-o) white matter axonal tracts. Panels e-g show higher magnification images of the distinctive expression pattern of NCX3 in the lateral tracts. Scale bars: 200μm (a); 50μm (h); 100μm (l); 20μm (b,c,d,i,j,k,m,n,o); 10μm (e,f); 5μm (g).

3. NCX3 co-expression with the myelin marker MBP and the axonal marker NF200 was reduced during EAE progression

To investigate the distribution of NCX3 within myelinated or demyelinated axons during EAE progression we performed confocal double immunofluorescence experiments of NCX3 antibody with the myelin marker MBP or the axonal marker NF200. Confocal double immunofluorescence experiments of NCX3 with the myelin marker MBP revealed a discrete overlapping of these two proteins in the white matter spinal cord of control animals (Fig. 10A,a-d) (Boscia et al., 2012; Gopalakrishnan et al., 2013). For instance, NCX3 punctate immunoreactivity frequently overlapped with myelin rings surrounding axons. Quantitative analysis of colocalized points revealed that the co-expression of these two proteins was significantly down-regulated at peak and chronic stages of EAE disease within demyelinated regions (Fig. 10B). This result was in line with the quantitative analysis of MBP fluorescence intensity. As shown in Figure 10C, a severe reduction of MBP during EAE progression was detected, being concentrated in plaque regions at the peak stage and with more disseminate appearance at chronic EAE stages (Fig. 10A, e-l). Quantitative confocal double immunofluorescence experiments of NCX3 with the axonal marker NF200 in spinal cord sections from control mice confirmed the presence of NCX3 exchanger within white matter axonal tracts. Quantification of colocalization studies within the range of 100 μm of distance from the slice border revealed a diverse amount of NCX3-positive axons in the lateral, anterior and posterior tracts. In particular, NCX3 was highly represented in NF200 positive axons of the lateral and the anterior tracts, but very little colocalization was detected along the posterior tracts (Fig. 11A).

According to the literature, the loss of intensity of NF200 immunoreactivity can be used as valuable marker to detect the axonal damage (Saatman et al., 1998; Yaghmai and Povlishock, 1992). Quantification analysis of NCX3 co-expression with the axonal marker NF200 within lateral tracts indicated that the number of NCX3-positive axons remained unchanged during the pre-onset stage (Fig. 11B; D,d-f) but they were significantly reduced during the peak stage (Fig. 11B; D,g-i) and this reduction remained unchanged at the chronic stages (Fig. 11B; D,j-l). Interestingly, the number of NF200-positive axons detected at EAE peak and chronic stages was higher than those observed co-expressing also the NCX3 exchanger (Fig. 11C;B). Although confocal experiments revealed that the co-expression of NCX3 within demyelinated axons was progressively down-regulated during EAE course, the NCX3 immunosignal became progressively and intensely evident within regions of both MBP and NF200 loss. This effect was particularly evident at chronic stage where a large number of cells with oligodendroglia-like morphology surrounding damaged axons were observed (Fig. 11D, p-u).

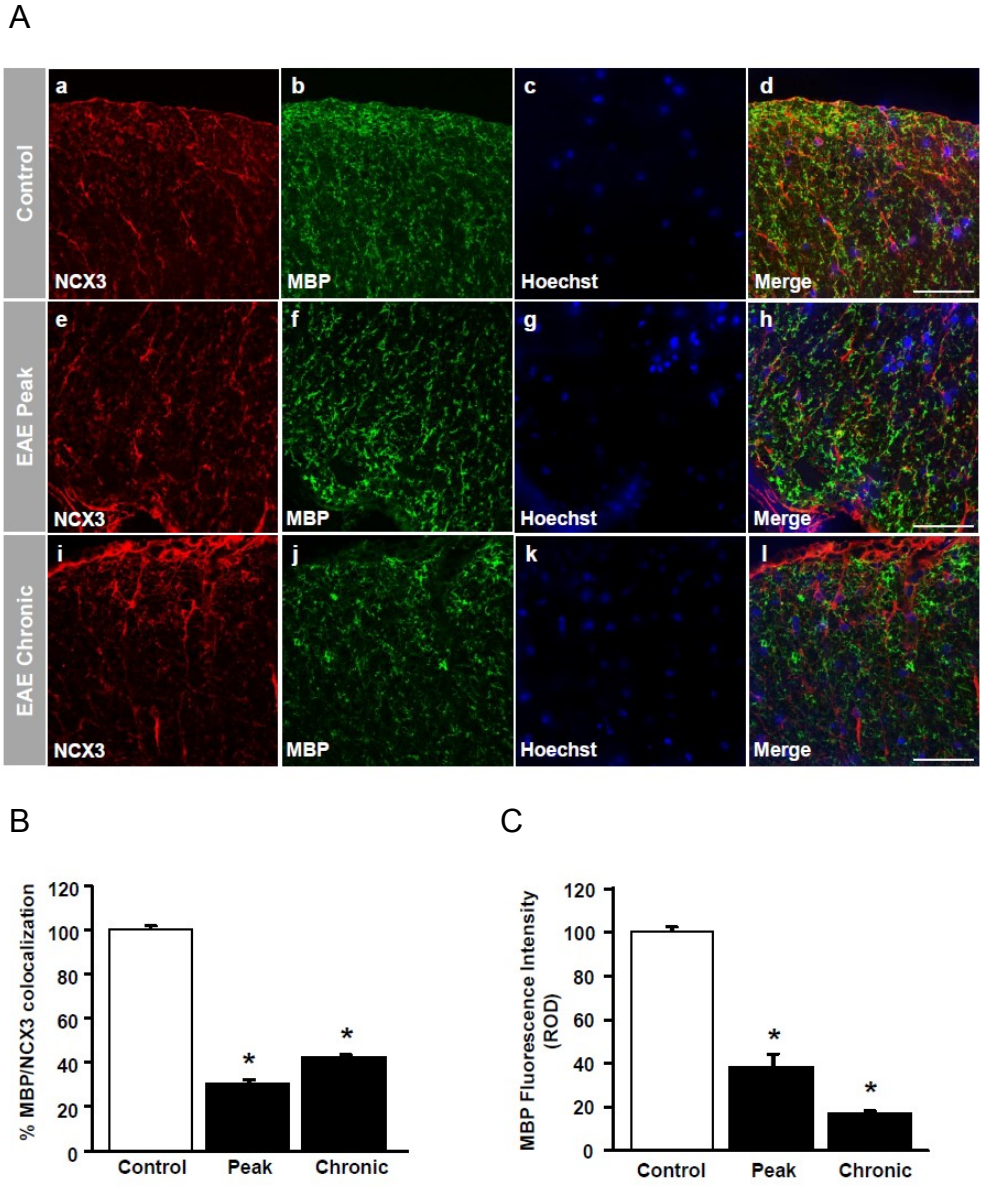


Figura 10. Co-expression of NCX3 with the myelin marker MBP in the white matter axonal tracts during EAE progression. (A) Confocal double-labelling experiments showing both NCX3 (red) and MBP (green) (a-l) in the white matter lateral tracts of control (a-d) and EAE affected-spinal cord during the Peak (e-h) and Chronic (i-l) stages. Scale bars: 50 μm (a-l) **(B)** Quantitative analysis of MBP/NCX3 colocalized points during EAE progression. The data were expressed as percentage of control. The values represent the means±S.E.M. (n=3-4). **P<0.05 versus* control. **(C)** Quantitative analysis of MBP fluorescence intensity during the EAE clinical course. The data were expressed as percentage of control. The values represent the means±S.E.M. (n=3-4). **P<0.05 versus* control.

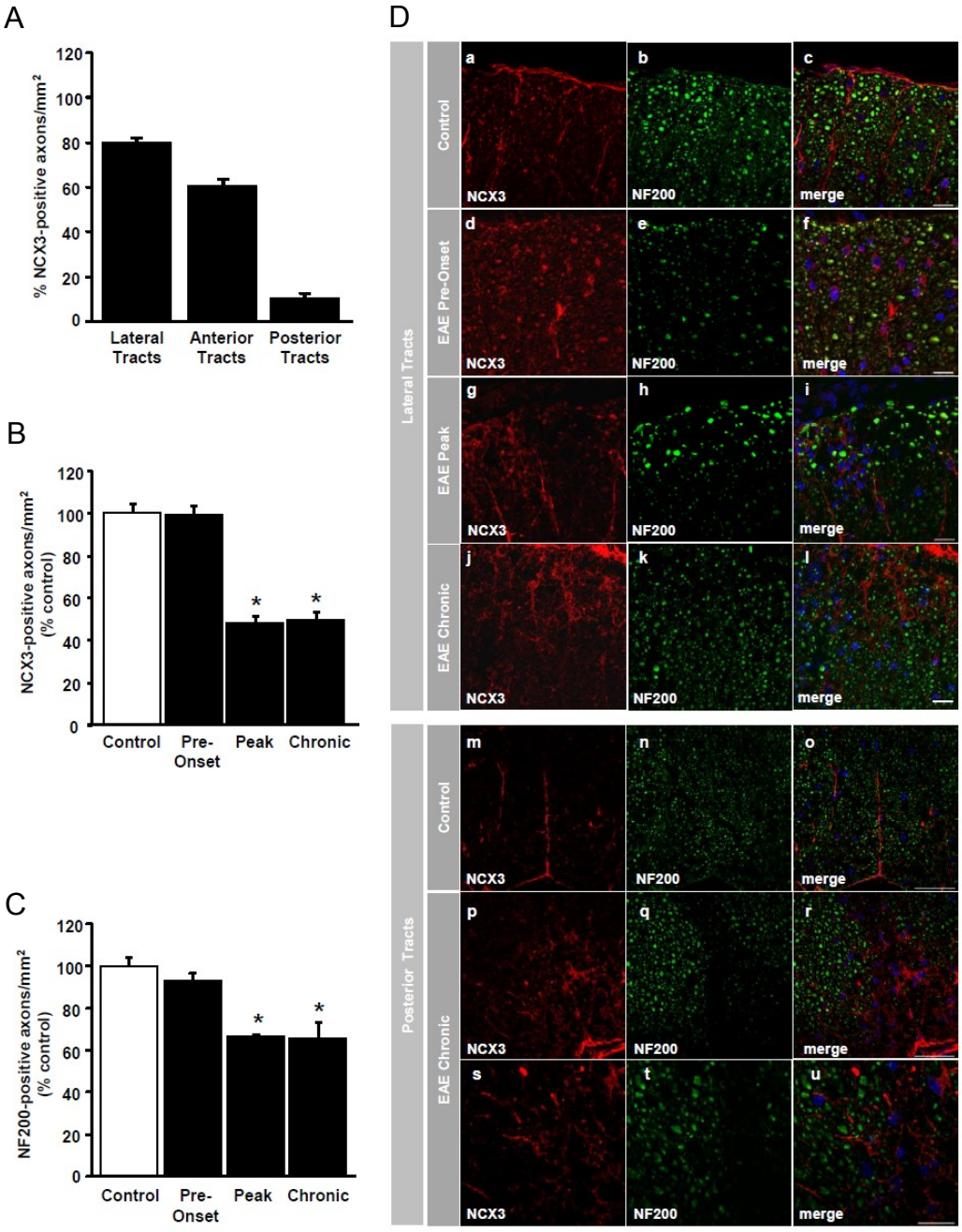


Figura 11. Co-expression of NCX3 with the axonal marker NF200 in the white matter axonal tracts during EAE progression. (A) Quantification of the percentage of NCX3-positive axons in lateral, anterior and posterior tracts of control mice. Analysis were performed within the range of 100 µm of distance from the slice border. The values represent the means±S.E.M. (n=3-4). **(B)** Quantitative analysis of NCX3-positive axons during EAE progression. The data were expressed as percentage of control. The values represent the means±S.E.M. (n=3-4). **P<0.05 versus* control. **(C)** Quantitative analysis of NF200-positive axons during EAE progression. The data were expressed as percentage of control. The values represent the means±S.E.M. (n=3-4). **P<0.05 versus* control. **(D)** Confocal double-labelling experiments showing both NCX3 (red) and NF200 (green) (a-u) immunosignal in the lateral (a-l) and posterior (m-u) tracts of control (a-c;m-o) and EAE-affected spinal cord at the Pre-Onset (d-f), Peak (g-i) and Chronic (j-l;p-u) stages. Scale bars: 20 µm (a-l); 50µm (m-r); 20µm (s-u).

4. NCX3 co-expression with the oligodendrocyte lineage markers NG2, GalC and CNPase was up-regulated at EAE chronic stage

To investigate whether NCX3 might be involved in the responses of oligodendroglial progenitors to EAE insult we performed colocalization experiments of NCX3 with anti-NG2, anti-GalC, and anti-CNPase antibodies at different time points during EAE progression.

Confocal analysis revealed that NG2 immunosignal was strongly up-regulated during the EAE course. In particular, at the pre-onset stage the anti-NG2 antibody sharply and intensely depicted positive cells with scattering distribution throughout the spinal cord (Fig.12A, b). At later time points, at peak and chronic stage of the disease, an intense expression of NG2 was observed within and around the demyelinated lesions (Fig.12A, c,d). Quantitative analysis revealed that the number of NG2-positive cells was significantly up-regulated during the EAE course when compared to control sections (Fig. 12B). Confocal double immunofluorescence experiments performed with anti-NCX3 and anti-NG2 antibodies revealed that NCX3 was largely co-expressed with NG2 in the white matter spinal cord of control animals (Fig. 12C, a-c). Quantification of NCX3-positive cells co-expressing NG2 within lateral tracts indicated that almost all NG2-positive cells co-expressed NCX3 at the pre-onset stage. By contrast, during the peak stage, although the number of NG2-positive cells was still significantly higher when compared to controls, the expression of NCX3 in these cells was reduced (Fig. 12D,B). Interestingly, a significant up-regulation of NCX3-NG2 double-labeled cells was detected at the chronic stage. At this latter time point, almost all the NG2-positive cells co-expressed NCX3 (Fig. 12C,j-l;D).

Confocally detected GalC immunoreactivity was strongly up-regulated and scattered detected along the lateral (Fig. 13A, a-d), anterior and posterior tracts (data not shown) during EAE course. Quantitative analysis revealed that the most significant increase in the number of GalC-positive cells was observed at the pre-onset stage. During the peak and the chronic stages the number of GalC-positive cells was reduced although significantly higher than those observed under control conditions (Fig. 13B). Confocal double immunofluorescence experiments performed with anti-NCX3 and anti-GalC antibodies revealed that several, although not all, GalC-positive cells co-expressed NCX3 in the white matter of control animals (Fig. 13C,a-c). Quantification of GalC-positive cells co-expressing NCX3 within lateral tracts indicated that almost all the GalC-positive cells co-expressed NCX3 at the pre-onset stage (Fig. 13C,d-f;D). By contrast, during the peak stage, although the number of GalC-positive cells was still significantly higher when compared to controls, the expression of NCX3 in these cells remained at the basal level (Fig. 13B;D). Interestingly, a significant up-regulation of NCX3-GalC double-labeled cells was detected at the chronic stage. At this latter time points, almost all the GalC-positive cells co-expressed NCX3 (Fig. 13C,j-o,D).

Confocal analysis performed with anti-CNPase antibody revealed that the number of CNPase-positive cells was significantly up-regulated only during the pre-onset and chronic stage of the disease (Fig. 14A,B). Interestingly, quantification of NCX3-CNPase double-labelled cells indicated that this isoform was intensely up-regulated in CNPase-positive cells both at pre-onset and chronic EAE stage (Fig. 14C,D). At this latter time point, NCX3 immunosignal was less intense expressed in the soma but much more evident along the processes of CNPase positive cells (14C,j-l).

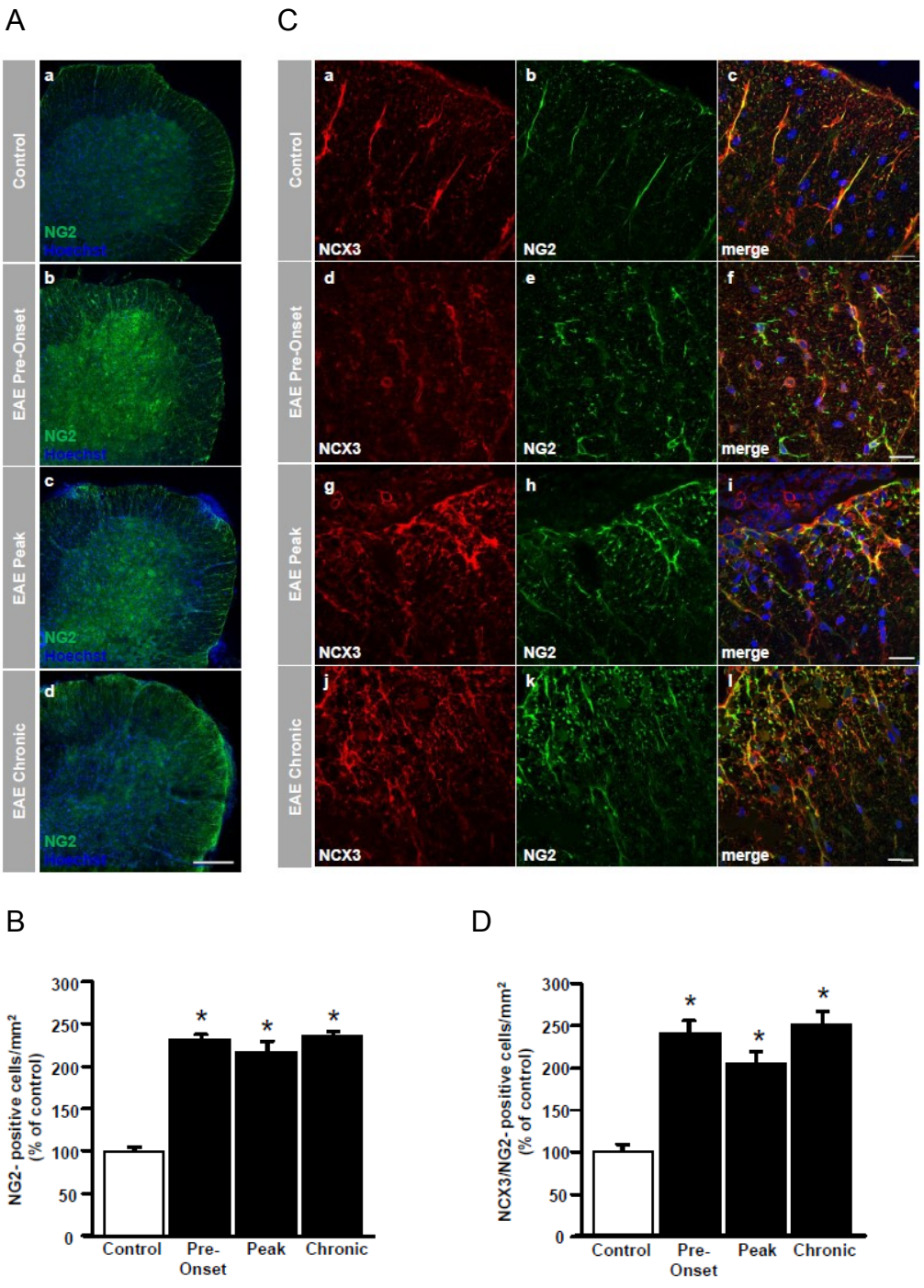


Figure 12. Co-expression of NCX3 with the oligodendrocyte lineage marker NG2 in the white matter during EAE progression. (A) Confocal microscopic images showing NG2 (green) immunosignal in control (a) and in EAE affected-spinal cord at the Pre-onset (b), Peak (c) and Chronic (d) stages. Scale bars: 200 µm (a-d) **(B)** Quantitative analysis of NG2-positive cells during EAE progression. The data were expressed as percentage of control. The values represent the means±S.E.M. (n=3-4). **P<0.05 versus* control. **(C)** Confocal double-labelling experiments showing both NCX3 (red) and NG2 (green) (a-l) immunosignal in the control (a-c) and in EAE-affected spinal cord at the Pre-Onset (d-f), Peak (g-i) and Chronic (j-l) stages. Scale bars: 20µm (a-l). **(D)** Quantitative analysis of NCX3-NG2 double-labelled cells during EAE progression. The data were expressed as percentage of control. The values represent the means±S.E.M. (n=3-4). **P<0.05 versus* control.

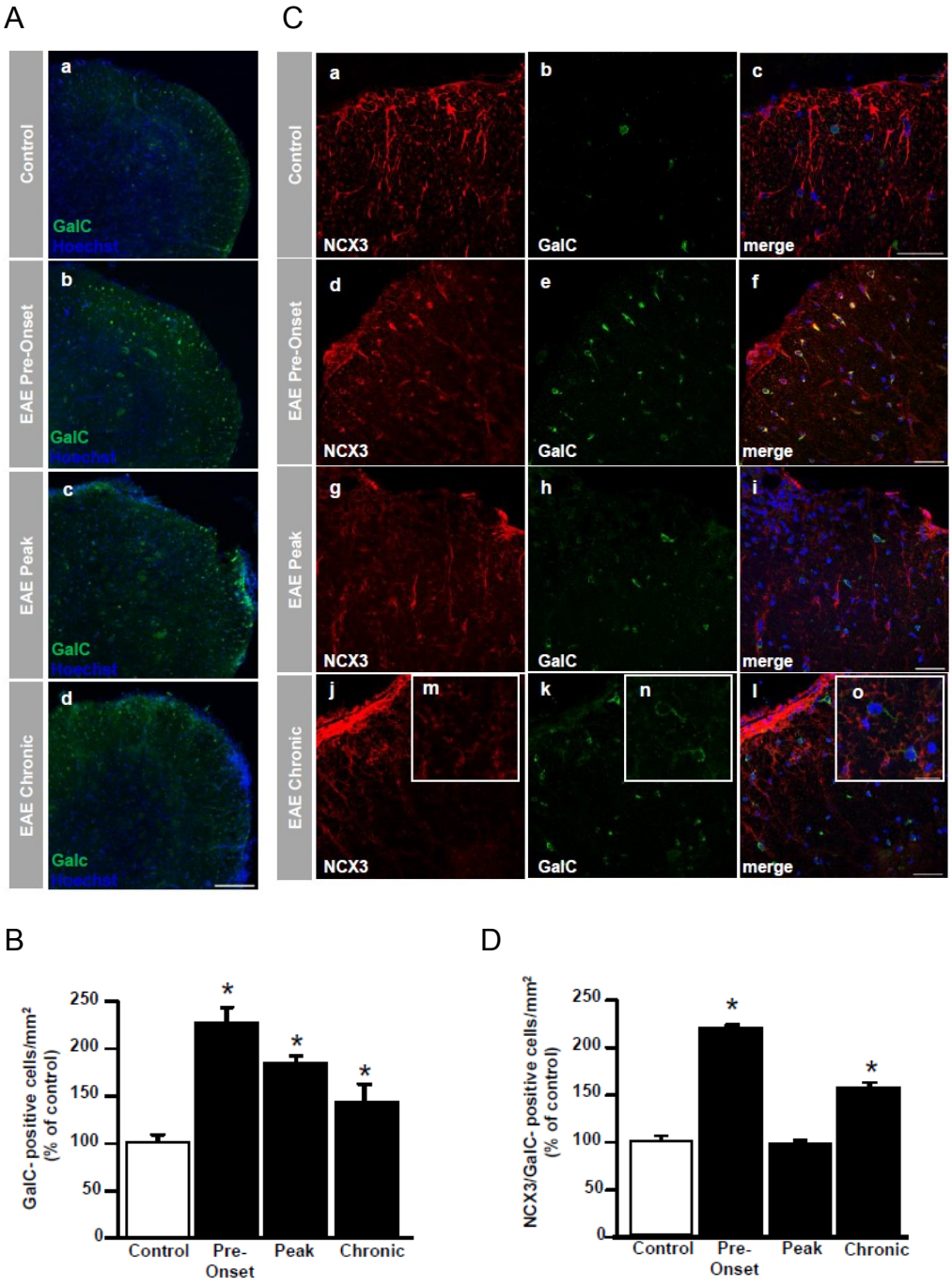


Figure 13. Co-expression of NCX3 with the oligodendrocyte lineage marker GalC in the white matter during EAE progression. (A) Confocal microscopic images showing GalC (green) immunosignal in control (a) and in EAE affected-spinal cord at the Pre-onset (b), Peak (c) and Chronic (d) stages. Scale bars: 200 µm (a-d). **(B)** Quantitative analysis of GalC-positive cells during EAE progression. The data were expressed as percentage of control. The values represent the means±S.E.M. (n=3-4). **P<0.05 versus* control. **(C)** Confocal double-labelling experiments showing both NCX3 (red) and GalC (green) (a-o) in the control (a-c) and in of EAE-affected spinal cord at the Pre-Onset (d-f), Peak (g-i) and Chronic (j-o) stages. Scale bars:50 µm (a-l); 20 µm (m-o). **(D)** Quantitative analysis of NCX3-GalC double-labelled cells during EAE progression. The data were expressed as percentage of control. The values represent the means±S.E.M. (n=3-4). **P<0.05 versus* control.

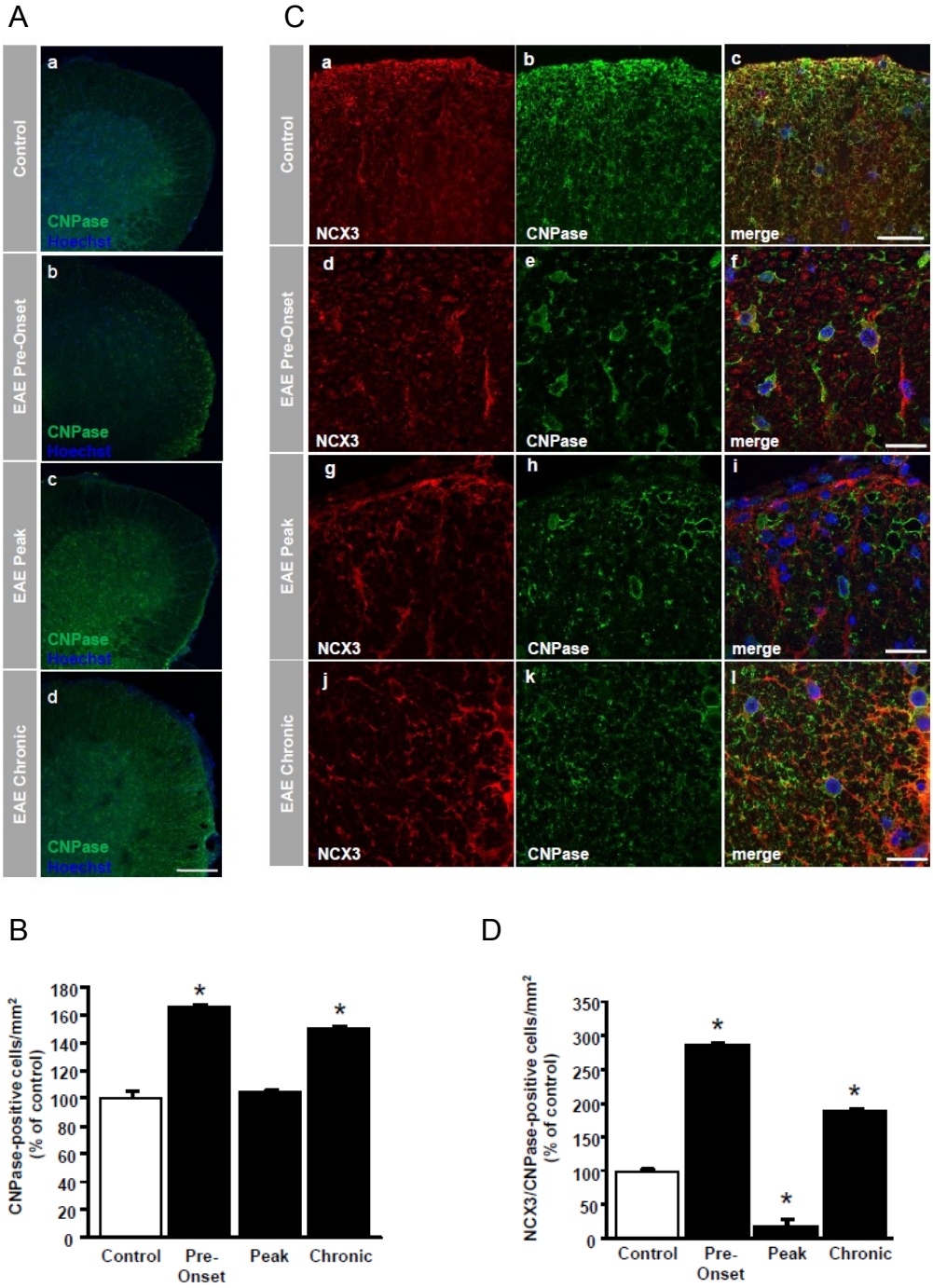


Figure 14. Co-expression of NCX3 with the oligodendrocyte lineage marker CNPase in the white matter during EAE progression. (A) Confocal microscopic images showing CNPase (green) immunosignal in control (a) and in EAE affected-spinal cord at the Pre-onset (b), Peak (c) and Chronic (d) stages. Scale bars: 200 μm (a-d). **(B)** Quantitative analysis of CNPase-positive cells during EAE progression. The data were expressed as percentage of control. The values represent the means±S.E.M. (n=3-4). **P<0.05 versus* control. **(C)** Confocal double-labelling experiments showing both NCX3 (red) and CNPase (green) (a-l) in the control (a-c) and in EAE-affected spinal cord at the Pre-Onset (d-f), Peak (g-i) and Chronic (j-l) stages. Scale bars:50μm (a-c); 20μm (d-l). **(D)** Quantitative analysis of NCX3-CNPase double-labelled cells during EAE progression. The data were expressed as percentage of control. The values represent the means±S.E.M. (n=3-4). **P<0.05 versus* control.

5. ncx3-/- mice exhibited increased susceptibility and impaired oligodendrocyte lineage responses to EAE disease

To determine whether NCX3 might have a role in controlling neuronal or oligodendroglial responses under demyelinating conditions, the EAE disease was induced in ncx3 congenic wild-type (ncx3+/+), heterozygous (ncx3+/-) and knockout (ncx3-/-) female mice. In all these animal groups the clinical score was assessed until day 40 after immunization, when the mice were sacrificed and subjected to immunohistochemical experiments to analyze demyelination, axonal damage and oligodendroglial responses.

Clinical assessment of EAE scores showed that both ncx3+/- and ncx3-/- mice displayed an earlier onset of symptoms that occurred 4±0.8 and 4±0.6 days after immunization respectively, when compared with their congenic wild-type littermates (10±0.5 dpi) (Fig. 15A,B). At the peak of the disease, the maximal severity of EAE was observed in ncx3+/- and ncx3-/- mice with a maximum clinical score of 4±0.3 and 4±0.5, respectively. During the chronic phase, until 40 dpi, ncx3+/- and ncx3-/- mice displayed higher level of disability when compared to ncx3 congenic wild-type mice (Fig. 15A,B). By contrast, there was no differences in the disease progression between C57BL/6 control and wild-type congenic ncx3 mice (data not shown). Furthermore, neither ncx3+/+, or ncx3+/-, or ncx3-/- mice showed any clinical signs following CFA injection (data not shown).

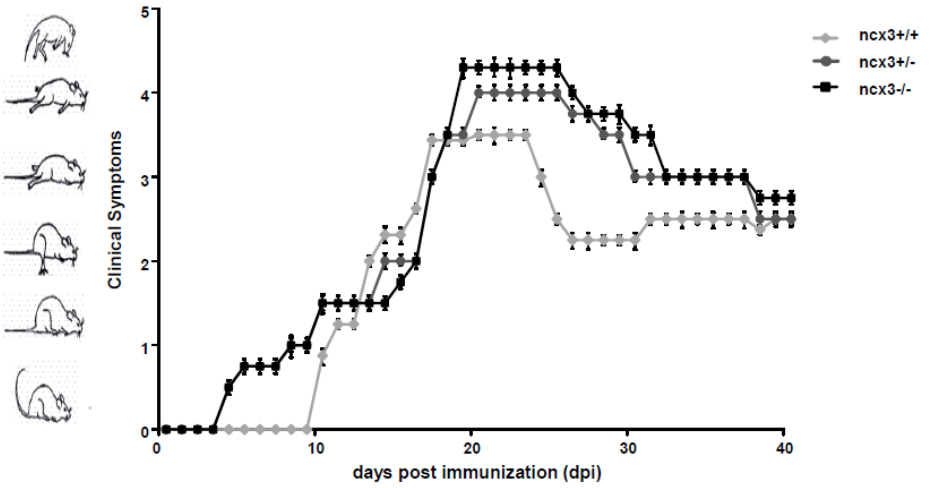
Ncx3-/- showed a dramatic reduction of intact myelin rings when compared to congenic wild-type at chronic stage. This was particular evident in white matter regions not affected by complete demyelination. In fact as showed in Figures 15C (a-d,l) the large majority of myelin rings observed in ncx3-/- sections appeared severely collapsed when compared to those observed in congenic animals.

Double labeling experiments performed with anti-MBP and anti-NF200 antibodies on congenic ncx3+/+ and ncx3-/- spinal cord sections at chronic stage of the disease revealed a significant reduction in both MBP and NF200 immunoreactivity (Fig. 15C,e-j). In addition, quantitative analysis revealed that spinal cord axon diameters of EAE ncx3-/- mice were smaller than those of congenic, wild-type, ncx3+/+ mice (Fig. 15C,m).

Quantitative immunohistochemical analysis of progenitor and premyelinating oligodendrocyte markers performed in both congenic and ncx3-/- mice at chronic stage indicated that a dramatic reduction of NG2-positive cells and CNPase-positive cells was observed in ncx3-/- mice if compared to congenic ncx3+/+ mice (Fig.16A;B).

Confocal double immunofluorescence experiments performed with anti-NG2 and anti-CNPase antibodies (Fig. 16C) revealed that several, although not all, NG2-positive cells co-expressed CNPase in the white matter of control animals. Interestingly, in ncx3-/- mice the NG2/CNPase co-expression was significantly down-regulated (Fig. 16C,c).

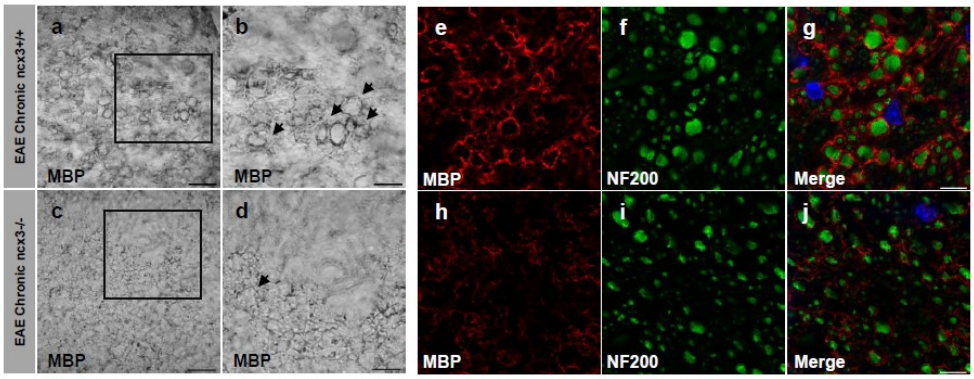
A



B

Experimental Group	Disease Onset	Disease Peak	Maximum Clinical Score
ncx3+/+	10 ± 0.5 dpi	17±1 dpi	3±0.1
ncx3+/-	4 ± 0.8 dpi	17±2 dpi	4±0.3
ncx3-/-	4 ± 0.6 dpi	18±2 dpi	4±0.5

C



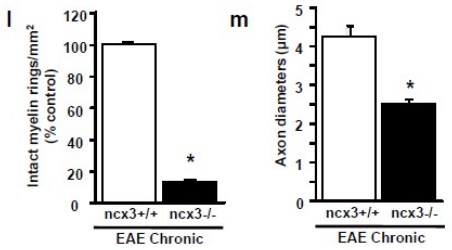


Figure 15. Assessment of EAE clinical scoring, myelin ring and axonal diameter in ncx3+/+ and ncx3-/- mice at EAE chronic stage (A,B) Graph and table showing the patterns of EAE clinical symptoms in the MOG_{35-55}-immunized ncx3 congenic wild-type (ncx3+/+), heterozygous (ncx3+/-) and knockout (ncx3-/-) mice, respectively. Results represent average (mean±S.E.M.) of the data obtained from 4 animals per group in three indipendent experimental sessions. **(C,**a-d**)** Single immunohistochemical experiments performed with anti-MBP antibody in the spinal cord of ncx3+/+ (a,b) and ncx3-/- (c,d) mice at the EAE chronic stage. Scale bars: 20µm (a,c); 10 µm (b,d). **(C,**e-j**)** Confocal double-labelling showing MBP (red) and NF200 (green) co-expression in the spinal cord of ncx3+/+ (e-g) and ncx3-/- (h-j) mice at the EAE chronic stage. Scale bars: 10µm (e-j). **(C,**l,m**)** Quantitative analysis of intact myelin rings (l) and axon diameter (m) in the spinal cord of ncx3+/+ and ncx3-/- mice at the EAE chronic stage. The data were expressed as percentage of control (l). The value represent the means±S.E.M. **P<0.05 versus* control.

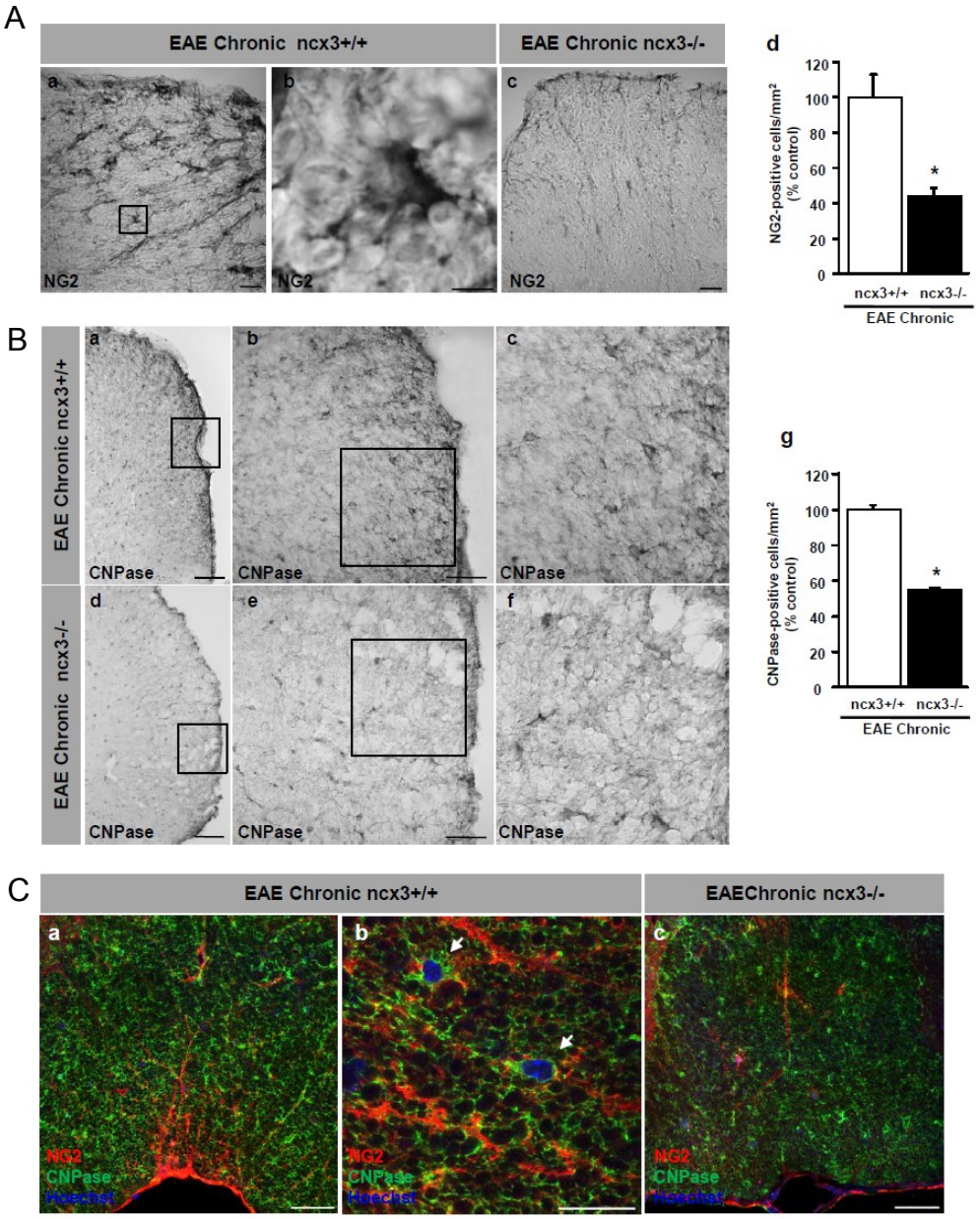


Figure 16. Analysis of NG2 and CNPase expression in the spinal cord of ncx3+/+ and in ncx3-/- mice at EAE chronic stage. (A,a-c**)** Single immunohistochemical experiments with anti-NG2 antibody performed in the spinal cord of ncx3+/+ (a,b) and ncx3-/- (c) mice at the EAE chronic stage. Scale bars: 20μm (a,c); 5μm (b). **(A,**d**)** Quantitative analysis of NG2-positive cells in white matter spinal cord of ncx3+/+ and ncx3-/- mice at the EAE chronic stage. The data were expressed as percentage of control. The values represent the means±S.E.M. **P<0.05 versus* control. **(B,**a-f**)** Single immunohistochemical experiments with anti-CNPase antibody performed in the spinal cord of ncx3+/+ (a-c) and ncx3-/- (d-f) mice at the EAE chronic stage. Scale bars: 20μm (a-f). **(B,g)** Quantitative analysis of CNPase-positive cells in white matter spinal cord of ncx3+/+ and ncx3-/- mice at the EAE chronic stage. The data were expressed as percentage of control. The values represent the means±S.E.M. **P<0.05 versus* control.**(C)** Confocal double-labelling showing NG2 (red) and CNPase (green) co-expression in the spinal cord of ncx3+/+ (a,b) and ncx3-/- (c) mice at the EAE chronic stage. Scale bars:50μm (a,c); 10μm (b).

V. DISCUSSION

The present study demonstrated that the Na^+/Ca^{2+} exchanger NCX3 is involved in the oligodendroglial responses under demyelinating conditions. In addition, NCX3 knockout mice exposed to Experimental Autoimmune Encephalomyelitis showed an impaired oligodendrocyte lineage responses and worsening of clinical symptoms.

Morphological analyses were performed at different time points during disease progression in order to assess inflammatory, neuronal and glial responses after MOG_{35-55}-induced EAE. In line with previous studies, this insult reproduced the key pathological features of MS including: demyelination, axonal loss and glial activation (Trafreshi et al., 2005).

Quantitative immunofluorescence experiments performed on EAE-affected spinal cord revealed that the number of cells positive for the oligodendrocyte precursor cell (OPCs) marker NG2 as well as the premyelinating oligodendrocyte markers GalC and CNPase, were significantly up-regulated during the EAE clinical course. Although the MOG_{35-55}-induced EAE might not be considered a model to study the remyelination process, many lines of evidence have shown that a common feature of experimental model of Multiple Sclerosis is the response of OPCs to repopulate the lesions site (Reynolds et al., 2002).

Biochemical analysis performed on spinal cord tissue homogenates revealed that NCX3 protein levels were progressively up-regulated during EAE progression, and this effect was more significant at EAE chronic stage. In order to evaluate the involvement of the NCX3 isoform during EAE demyelinating conditions, in the current study we provided a clear picture of NCX3 expression and distribution within neurons and oligodendrocytes of the white matter spinal cord at different time points during EAE disease. Quantitative confocal double immunofluorescence experiments showed that, although the co-expression of NCX3 with

both the myelin protein marker MBP and the axonal marker NF200 was significantly down-regulated, the co-expression of NCX3 with the oligodendrocyte lineage markers, NG2, GalC and CNPase was up-regulated during EAE progression. These results suggested that NCX3 isoform, by modulating Na^+ and Ca^{2+} homeostasis, might be involved in oligodendroglial responses observed during EAE clinical course.

Consistently with the possible role of NCX3 in OPCs responses, in a paper recently published by our research group, it has been demonstrated that calcium signaling mediated by NCX3 has a crucial role in the progression of OPCs into mature oligodendrocytes (Boscia et al., 2012).

In support of our hypothesis we found that the response of OPCs cells, usually observed under demyelinating conditions, are compromised by the ablation of NCX3. In fact, analysis of NG2 and CNPase expression revealed that NCX3 knockout mice showed a reduced number of NG2- and CNPase-positive cells in the white matter spinal cord at the EAE chronic stage when compared to wild-type EAE-affected mice.

Antibodies to the NG2 have proved exceedingly useful in following and quantitating the response of endogenous OPCs to demyelination (Reynolds et al., 2002). In addition, lines of evidence have shown that NG2-expressing OPCs responded to the inflammatory demyelination in MOG-induced EAE model by becoming reactive and increasing in number in a very focal manner. In accordance with our findings, evidence of NG2-positive OPCs in lesioned areas beginning to express the oligodendrocyte marker CNPase was also seen (Reynolds et al., 2002). These observations further support our hypothesis indicating that, under demyelinating conditions, the NCX3 exchanger might have a relevant role in the stimulation of OPCs responses.

Interestingly, the reduced recovery response observed in NCX3 knockout mice at EAE chronic stage was accompanied by

morphological alterations of myelin and axons and by a worsening of clinical symptoms. In particular, in NCX3 knockout mice the amount of morphologically intact ring-like myelin structures was reduced when compared to NCX3 congenic wild-type mice at EAE chronic stage. This was particular evident in white matter regions not affected by complete demyelination; the large majority of myelin rings observed in NCX3 knockout sections appeared severely collapsed when compared to those observed in congenic animals. In addition, NCX3 knockout mice exposed to EAE displayed a significant reduction of axonal diameter, this suggesting an increased axonal damage in EAE NCX3 knockout mice.

It has been recently demonstrated that axonal degeneration contributes to the development of non-remitting neurological deficits and disability in Multiple Sclerosis, but the molecular mechanisms that underlie axonal loss in Multiple Sclerosis are not clearly understood. Although the Na^+/Ca^{2+} exchanger appeared to be involved, its precise role in axonal degeneration has not been resolved. Waxam and co-workers (Craner et al., 2004a) have shown that NCX co-localizes with Nav1.6 in the injured axons in the spinal cord of mice with EAE (Craner et al., 2004b). In particular, Craner et al. (2004a) have demonstrated the up-regulated expression of Nav1.2 and Nav1.6 along extensive regions of demyelinated axons in EAE. Several lines of evidence suggest that Nav1.6 contributes to the persistent current that drives reverse NCX in injured axons in Multiple Sclerosis. The proposed mechanism involves massive sodium influx into axons that triggers reverse operation of the exchanger and subsequent influx of damaging levels of intra-axonal calcium that is associated with axonal injury (Craner et al., 2004a).

Our results suggest that the NCX3 isoform might be not primarily involved in axonal degeneration. In fact, the findings of the present study revealed that the axonal expression of this exchanger isoform

was significantly reduced during EAE progression and, more importantly, NCX3 knockout mice displayed enhanced susceptibility to the disease and more severe neurological deficits. Further studies are, indeed, required to understand whether some other isoforms of the Na^+/Ca^{2+} exchanger family might be involved.

In addition, it is possible to speculate that the morphological alterations observed in myelin and axons of NCX3 knockout mice might be a direct consequence of the reduced oligodendroglial responses observed during the recovery phase. Consistently with the neuroprotective role of this exchanger, recent evidence point out to NCX3 isoform as a new potential therapeutic target for neuroprotection. Indeed, mice lacking NCX3 by silencing or transgenic approaches, show an enhanced cellular vulnerability to hypoxic-ischemic insults (Secondo et al., 2007) and a worsening of the infarct area after stroke (Pignataro et al., 2004; Molinaro et al., 2008). More recently, NCX3 has also been proposed as a new molecular effector involved in the neuroprotective effect of ischemic post-conditioning (Pignataro et al., 2011). In agreement with the beneficial role of this exchanger, it has been demonstrated that NCX3-deficient mice show skeletal muscle fiber necrosis and impaired neuromuscular transmission, which is clinically associated with reduced motor activity, weakness of forelimb muscles, and fatigability (Sokolow et al., 2004).

Overall, our findings suggest that calcium signaling mediated by NCX3 may be a key contributing factor to the impaired oligodendrocyte lineage responses that characterizes the EAE demyelinating condition. Further studies are needed to understand the role of NCX3 during remyelination processes.

VI. REFERENCES

Amoroso S., De Maio M., Russo G.M., Catalano A., Bassi A., Montagnani S., Di Renzo G.F., and Annunziato L. (1997). Pharmacological evidence that the activation of the Na+/Ca2+ exchanger protects C6 glioma cells during chemical hypoxia. Br J Pharmacol 121:303–309.

Amoroso S., Sensi S., Di Renzo G.F., and Annunziato L. (1993). Inhibition of the Na+/Ca2+ exchange enhances anoxia and glucopenia-induced 3H-aspartate release in hippocampal slices. J Pharmacol Exp Ther 264:515–520.

Amoroso S., Taglialatela M., Canzoniero L.M., Cragoe E.J. Jr., di Renzo G., and Annunziato L. (1990). Possible involvement of Ca2+, protein kinase C and Na+/H+ antiporter in insulin-induced endogenous dopamine release from tuberoinfundibular neurons. Life Sci 46:885–894.

Amoroso S., Tortiglione A., Secondo A., Catalano A., Montagnani S., Di Renzo G., and Annunziato L. (2000). Sodium nitroprusside prevents chemical hypoxia-induced cell death through iron ions stimulating the activity of the Na+/Ca2+ exchanger in C6 glioma cells. J Neurochem 744:1505–1513.

Andreeva N., Khodorov B., Stelmashook E., Cragoe E., and Victorov I (1991). Inhibition of Na+/Ca2+ exchange enhances delayed neuronal death elicited by glutamate in cerebellar granule cell cultures. Brain Res 548:322–325.

Annunziato L., Pannaccione A., Cataldi M., Secondo A., Castaldo P., Di Renzo G., and Taglialatela M. (2002). Modulation of ion channels by reactive oxygen and nitrogen species: a pathophysiological role in brain aging? Neurobiol Aging 23:819–834.

Annunziato L., Pignataro G., Di Renzo G.F. (2004). Pharmacology of brain Na+/Ca2+ exchanger: from molecular biology to therapeutic perspectives. Pharmacol Rev 56:633-654

Annunziato L., Boscia F., Pignataro G.,(2013). Ionic transporter activity in astrocytes, microglia, and oligodendrocytes during brain ischemia. J Cereb Blood Flow Metab. Jul;33(7):969-82. doi:10.1038/jcbfm..44. Epub. Apr 3. Review.

Atherton J., Kurbatskaya K., Bondulich M., Croft C. L., Garwood C. J., Chhabra R.,Wray S., Jeromin A., Hanger D.P. and Noble W. (2014). Calpain cleavage and inactivation of the sodium calcium exchanger-3 occur downstream of Aβ in Alzheimer's disease. Aging Cell 13(1):49-59.

Baker P.F. and Blaustein M.P. (1968). Sodium-dependent uptake of calcium by crab nerve. Biochim Biophys Acta 150:167–170.

Baker P.F., Blaustein M.P., Hodgkin A.L., and Steinhardt R.A. (1969). The influence of calcium on sodium efflux in squid axons. J Physiol 200:431–458.

Barnett M.H., Prineas J.W., (2004). Relapsing and remitting multiple sclerosis: pathology of the newly forming lesion. Ann Neurol 55:458–468.

Bauer J.A., (1994). Reduced coronary vasodilator function after myocardial infarction N Engl J Med. 1994 Dec 8;331(23):1590-1.

Benveniste H., Drejer J., Schousboe A., Diemer N.H. (1984). Elevation of the extracellular concentrations of glutamate and aspartate in rat hippocampus during transient cerebral ischemia monitored by intracerebral microdialysis. JNeurochem. 43(5):1369-74.

Bickler P.E., Buck L.T., Hansen B.M.,(1994). Effects of isoflurane and hypothermia on glutamate receptor-mediated calcium influx in brain slices.Anesthesiology.Bickler BrainRes. Dec5;665(2):269-
76.81(6):1461-9.

Bignami A., Eng L.F., Dahl D., Uyeda C.T., (1972). Localization of the glial fibrillary acidic protein in astrocytes by immunofluorescence.Brain Res 43:429–435.

Black J.A., Dusart I., Sotelo C., Waxman S.G., (2002). Axotomy does not up-regulate expression of sodium channel Na(v)1.8 in Purkinje cells.Brain Res Mol Brain Res. 2002 May 30;101(1-2):126-31.

Black J.A., NewcombeJ., Trapp B.D., Waxman S.G., (2007). Sodium channel expression within chronic multiple sclerosis plaques. J Neuropathol Exp Neurol. Sep;66(9):828-37.

Blakemore W.F. and Franklin R.J. (2008). Remyelination in experimental models of toxin-induced demyelination. Curr Top Microbiol Immunol;318:193-212.

Blaustein M.P. and Lederer W.J., (1999). Sodium/calcium exchange: its physiological implications. Physiol Rev;79:763–854.

Bo L., Mork S., Kong P.A., Nyland H., Pardo C.A., Trapp B.D., (1994). Detection of MHC class II-antigens on macrophages and microglia, but not on astrocytes and endothelia in active multiple sclerosis lesions. J Neuroimmunol 51:135–146.

Boscia F., Gala R., Pannaccione A., Secondo A., Scorziello A., Di Renzo G., Annunziato L.,(2009). NCX1 expression and functional activity increase in microglia invading the infarct core. Stroke. Nov; 40(11):3608-17.

Boscia F., D'Avanzo C., Pannaccione A., Secondo A., Casamassa A., Formisano L., Guida N., Sokolow S., Herchuelz A., Annunziato L. (2012).Silencing or knocking out the Na+/Ca2+ exchanger-3 (NCX3) impairs oligodendrocyte differentiation. Cell Death Differ.19(4):562-72.

Boscia F., D'Avanzo C., Pannaccione A., Secondo A., Casamassa A., Formisano L., Guida N., Scorziello A., Di Renzo G., Annunziato L., (2013). New roles of NCX in glial cells: activation of microglia in ischemia and differentiation of oligodendrocytes. Adv Exp Med Biol;961:307-16.

Bostock H., Sears T.A., (1976). Continuous conduction in demyelinated mammalian nerve fibers. Nature. 263(5580):786-7.

Bostock H., Sears T.A.,(1978). The internodal axon membrane: electrical excitability and continuous conduction in segmental demyelination.J Physiol. 280:273-301.

Canitano A., Papa M., Boscia F., Castaldo P., Sellitti S., Taglialatela M. and Annunziato L., (2002). Brain distribution of the Na+/Ca2+ exchanger-encoding genes NCX1, NCX2 and NCX3 and their related proteins in the central nervous system. Ann N Y Acad Sci. 976:394–404.

Canzoniero L.M., Rossi A., Taglialatela M., Amoroso S., Annunziato L. and Di Renzo G.F.,(1992). The Na+/Ca2+ exchanger activity in cerebrocortical nerve endings is reduced in old compared to young and mature rats when it operates as a Ca2+ influx or efflux pathway. Biochim Biophys Acta. 1107:175–178.

Charcot J. M., Hoché G., Sanders L.J.,(1868). On some arthropathies apparently related to a lesion of the brain or spinal cord, J Am Podiatr Med Assoc. 82(8):403-11

Chen Y. and Swanson R.A. (2003) Astrocytes and brain injury. J Cereb Blood Flow Metab. 23:137-149Cho D.H., Chung K.C., Jeong S.S., Park M.Y.,(2000). Potentiometric behavior of N,N,N',N'-tetrabenzylmethylenediamine-based hydrogen ion-selective electrodes. Talanta. 51(4):761-7.

Choi D.W., (1988). Calcium-mediated neurotoxicity: relationship to specific channel types and role in ischemic damage. Trends Neurosci. 11:465–469.

Compston A., Coles A., (2008). Multiple sclerosis. Lancet. 372(9648):1502-17.

Constantinescu C.S., Hilliard B., Ventura E., Wysocka M., ShoweL., Lavi E., Fujioka T., Scott P., Trinchieri G., Rostami A.,(2001). Modulation of susceptibility and resistance to an autoimmune model of multiple sclerosis in prototypically susceptible and resistant strains by neutralization of interleukin-12 and interleukin-4, respectively. Clin Immunol. 98(1):23-30.

Constantinescu C.S., Farooqi N., O'Brien K., Gran B., (2011). Experimental autoimmune encephalomyelitis (EAE) as a model for multiple sclerosis(MS). Br J Pharmacol. 164(4):1079-106.

Craner M.J., Newcombe J., Black J.A., Hartle C., Cuzner M.L., Waxman S.G.,(2004a). Molecular changes in neurons in multiple sclerosis: altered axonal expression of Nav1.2 and Nav1.6 sodium channels and Na+/Ca2+ exchanger. Proc Natl Acad Sci U S A. 2004 May 25;101(21):8168-73.

Craner M.J., Hains B.C., Lo A.C., Black J.A., Waxman S.G., (2004b).Co-localization of sodium channel Nav1.6 and the sodium-calcium exchanger at sites of axonal injury in the spinal cord in EAE. Brain. 127(Pt 2):294-303.

Crawford A.H., Chambers C. and Franklin R.J. (2013). Remyelination: the true regeneration of the central nervous system. J Comp Pathol.149(2-3):242-54.

Czyz A. and Kiedrowski L., (2002). In depolarized and glucose-deprived neurons, Na+ influx reverses plasmalemmal K+-dependent and K+-independent Na+/Ca2+ exchangersand contributes to NMDA excitotoxicity. J Neurochem. 83:1321–1328.

Day M., (2005). Histopatology of EAE. Experimental Models of Multiple Sclerosis. Springer:New York, pp.25-43.

de la Pena P. and Reeves J.P., (1987) Inhibition and activation of Na+/Ca2+ exchange activity by quinacrine. Am J Physiol. 252:C24–C29.

Denic A., Johnson A.J., Bieber A.J., Warrington A.E., Rodriguez M. and Pirko I. (2011). The relevance of animal models in multiple sclerosis research. Pathophysiology. 18(1):21-9.

Derwenskus J. (2011). Current disease-modifying treatment of multiple sclerosis. Mt Sinai J Med. 78(2):161-75

Di Renzo G., Amoroso S., Bassi A., Fatatis A., Cataldi M., Colao A.M., Lombardi G. and Annunziato L., (1995). Role of the Na+/Ca2+ and

Na+-H+ antiporters in prolactin release from anterior pituitary cells in primary culture. Eur J Pharmacol. 294:11–15.

Dubinsky J.M. and Rothman S.M., (1991). Intracellular calcium concentrations during "chemical hypoxia" and excitotoxic neuronal injury. Neurosci. 11(8):2545-51.

Ellwardt E. and Zipp F., (2014). Molecular mechanisms linking neuroinflammation and neurodegeneration in MS. Exp Neurol. doi: 10.1016.

Erecińska M. and Silver I.A.,(1992). Relationship between ions and energy metabolism: cerebral calcium movements during ischaemia and subsequent recovery. Can J Physiol Pharmacol. 70 Suppl:S190-3.

Eriksson K.S., Sergeeva O., Brown R.E. and Haas H.L., (2001a). Orexin/hypocretin excites the histaminergic neurons of the tuberomammillary nucleus. J Neurosci. 21:9273–9279.

Eriksson K.S., Stevens D.R. and Haas H.L., (2001b). Serotonin excites tuberomammillary neurons by activation of Na+/Ca2+ exchange. Neuropharmacology. 40(3):345-51.

Fancy S.P., Zhao C. and Franklin R.J. (2004). Increased expression of Nkx2.2 and Olig2 identifies reactive oligodendrocyte progenitor cells responding to demyelination in the adult CNS. Mol Cell Neurosci. 27(3):247-54.

Farooqi N., Gran B. and Constantinescu C.S., (2010). Are current disease-modifying therapeutics in multiple sclerosis justified on the basis of studies in experimental autoimmune encephalomyelitis? JNeurochem. 115(4):829-44.

Ferguson B., Matyszak M.K., Esiri M.M. and Perry V.H., (1997). Axonal damage in acute multiple sclerosis lesions. Brain. 120(Pt 3):393–399

Finkbeiner S.M., (1993). Glial calcium. Glia. 9:83-104

Franklin R.J. and ffrench-Constant C., (2008). Remyelination in the CNS: from biology to therapy. Nat Rev Neurosci. 9(11):839-55.

Furukawa K. and Mattson M.P., (1995). Cytochalasins protect hippocampal neurons against amyloid beta-peptide toxicity: evidence that actin depolymerization suppresses Ca2+ influx. J Neurochem. 65:1061–1068.

Garthwaite G., Goodwin D.A., Batchelor A.M., Leeming K. and Garthwaite J. (2002). Nitric oxide toxicity in CNS white matter: an in vitro study using rat optic nerve. Neuroscience.109(1):145-55.

Gensert J.M. and Goldman J.E.,(1997). Endogenous progenitors remyelinate demyelinated axons in the adult CNS. Neuron. 19(1):197-203.

Giaume C., Kirchhoff F., Matute C., Reichenbach A. and Verkhratsky A., (2007). Glia: the fulcrum of brain diseases. Cell Death Differ. 14(7):1324-35.

Ginhoux F., Greter M., Leboeuf M., Nandi S., See P., Gokhan S., Mehler M.F., Conway S.J., Ng L.G., Stanley E.R., Samokhvalov I.M. and Merad M., (2010). Fate mapping analysis reveals that adult microglia derive from primitive macrophages. Science. 330(6005):841-5.

Goldman W.F., Yarowsky P.J., Juhaszova M., Krueger B.K., Blaustein M.P. (1994) Sodium/calcium exchange in rat cortical astrocytes. J Neurosci. 14:5834-5843

Gopalakrishnan G., Awasthi A., Belkaid W., De Faria O. Jr., Liazoghli D., Colman D.R. and Dhaunchak A.S., (2013). Lipidome and proteome map of myelin membranes. J Neurosci Res. 91(3):321-34.

Gran B., O' Brien K., Fitzgerald D. and Rostami A., (2007). Experomental Autoimmune Encephalomyelitis. Handbook of Neurochemistry and Molecular Neurobiology A. Lajtha. Springer: Heidelberg, p.19

Hafler D.A.,(2004). Multiple sclerosis. J Clin Invest. 113(6):788-94.

Hauser S.L., Reinherz E.L., Hoban C.J., Schlossman S.F. and Weiner H.L.I., (1983). Immunoregulatory T-cells and lymphocytotoxic antibodies in active multiple sclerosis: weekly analysis over a six-month period. Ann Neurol. 13(4):418-25.

Hauser S.L., Reinherz E.L., Hoban C.J., Schlossman S.F. and Weiner H.L., (1983). CSF cells in multiple sclerosis: monoclonal antibody analysis and relationship to peripheral blood T-cell subsets.Neurology. 33(5):575-9.

He Z., Tong Q., Quednau B.D. Philipson K.D. and Hilgemann D.W,. (1998). Cloning, expression, and characterization of the squid Na+/Ca2+ exchanger (NCX-SQ1). J Gen Physiol. 111:857-873

Henderson S.A., Goldhaber J.I., So J.M., Han T., Motter C., Ngo A., Chantawansri C., Ritter M.R., Friedlander M., Nicoll D.A., Frank J.S., Jordan M.C., Roos K.P., Ross R.S. and Philipson K.D. (2004). Functional adult myocardium in the absence of Na+-Ca2+ exchange: cardiac-specific knockout of NCX1. Circ Res. 95(6):604-11.

Hilgemann D.W. (1990). Regulation and deregulation of cardiac Na+/Ca2+ exchange in giant excised sarcolemmal membrane patches. Nature. 344:242–245.

Hill R.A., Patel K.D., Medved J., Reiss A.M. and Nishiyama A. (2013). NG2 cells in white matter but not gray matter proliferate in response to PDGF. J Neurosci.33(36):14558-66.

Hofstetter H.H., Shive C.L. and Forsthuber T.G. (2002). Pertussis toxin modulates the immune response to neuroantigens injected in incomplete Freund's adjuvant: induction of Th1 cells and experimental autoimmune encephalomyelitis in the presence of high frequencies of Th2 cells. J Immunol. 169(1):117-25.

Holgado A. and Beauge L., (1996). Effects of external monovalent cations on Na+/Ca2+ exchange in cultured rat glial cells. Ann N Y Acad Sci. 779:279-281.

Iwamoto T., Watano T., and Shigekawa M. (1996). A novel isothiourea derivative selectively inhibits the reverse mode of Na+/Ca2+ exchange in cells expressing NCX1. J Biol Chem. 271:22391–22397.

Jakovcevski I. and Zecevic N. (2005). Olig transcription factors are expressed in oligodendrocyte and neuronal cells in human fetal CNS. J Neurosci. 25(44):10064-73.

Jakovcevski I., Filipovic R., Mo Z., Rakic S. and Zecevic N., (2009). Oligodendrocyte development and the onset of myelination in the human fetal brain. Front Neuroanat. 2009. 3:5.

Jeon D., Chu K., Kim Y., Yoon B., and Shin H.S., (2003a). Na+/Ca2+ exchanger 2 protects cell death after focal cerebral ischemia. Society for Neuroscience. 308.10.

Kessaris N., Fogarty M., Iannarelli P., Grist M., Wegner M. and Richardson W.D., (2006). Competing waves of oligodendrocytes in the forebrain and postnatal elimination of an embryonic lineage.Nat Neurosci. 9(2):173-9.

Kiedrowski L., (1999). N-methyl-D-aspartate excitotoxicity: relationships among plasma membrane potential, Na+/Ca2+ exchange, mitochondrial Ca2+ overload, and cytoplasmic concentrations of Ca2+, H+, and K+. Mol Pharmacol. 56:619–632.

Koushik S.V., Wang J., Rogers R., Moskophidis D., Lambert N.A., Creazzo T.L. and Conway S.J., (2001). Targeted inactivation of the sodium-calcium exchanger (Ncx1) results in the lack of a heartbeat and abnormal myofibrillar organization. Faseb J. 15(7):1209-11.

Kristián T., Ouyang Y. and Siesjö B.K.,(1996). Calcium-induced neuronal cell death in vivo and in vitro: are the pathophysiologic mechanisms different? Adv Neurol. 71:107-113.

Kristián T. and Siesjö B.K.,(1996). Calcium-related damage in ischemia. Life Sci. 59(5-6):357-67.

Kirischuk S., Kettenmann H. and Verkhratsky A. (1997). Na+/Ca2+ exchanger modulates kainate-triggered Ca2+ signaling in Bergmann glial cells in situ. Faseb J. 11:566-572

Konrad K. and Eickhoff S.B.,(2010). Is the ADHD brain wired differently? A review on structural and functional connectivity in attention deficit hyperactivity disorder. Hum Brain Mapp. 31(6):904-16.

Kornek B. and Lassmann H. (1999). Axonal pathology in multiple sclerosis. A historical note. Brain Pathol. 9(4):651-6.

Kornek B., Storch M.K., Weissert R., Wallstroem E., Stefferl A., Olsson T., Linington C., Schmidbauer M. and Lassmann H.(2000). Multiple sclerosis and chronic autoimmune encephalomyelitis: a comparative quantitative study of axonal injury in active, inactive, and remyelinated lesions. Am J Pathol. 157(1):267-76.

Kucharova K., Chang Y., Boor A., Yong V.W. and Stallcup W.B. (2011). Reduced inflammation accompanies diminished myelin damage and repair in the NG2 null mouse spinal cord. J Neuroinflammation. 8:158.

Ifuku M., Färber K., Okuno Y., Yamakawa Y., Miyamoto T., Nolte C., Merrino V.F., Kita S., Iwamoto T., Komuro I., Wang B., Cheung G., Ishikawa E., Ooboshi H., Bader M., Wada K., Kettenmann H. and Noda M., (2007). Bradykinin-induced microglial migration mediated by B1-bradykinin receptors depends on Ca2+ influx via reverse-mode activity of the Na+/Ca2+ exchanger. J Neurosci. 27(48):13065-73.

Lassmann H., Raine C.S., Antel J. and Prineas J.W. (1998). Immunopathology of multiple sclerosis: report on an international meeting held at the Institute of Neurology of the University of Vienna. J Neuroimmunol. 86:213–7.

Lars Bo, Esiri M., Evangelou N. and Kuhlmann T. (2013). Myelin Repair and Neuroprotection in Multiple Sclerosis. Springer.

Lalive P.H., Neuhaus O., Benkhoucha M., Burger D., Hohlfeld R., Zamvil S.S. and Weber M.S. (2011). Glatiramer acetate in the treatment

of multiple sclerosis: emerging concepts regarding its mechanism of action. CNS Drugs 25(5):401-414.

Lee S.L., Yu A.S. and Lytton J., (1994). Tissue-specific expression of Na+/Ca2+ exchanger isoforms. J Biol Chem. 269:14849–14852.

Levine J.M. and Reynolds R., (1999). Activation and proliferation of endogenous oligodendrocyte precursor cells during ethidium bromide-induced demyelination. Exp Neurol. 160:333–347

Li S., Jiang Q. and Stys P.K., (2000). Important role of reverse Na+/Ca2+ exchange in spinal cord white matter injury at physiological temperature. J Neurophysiol. 84:1116–1119.

Li Z., Matsuoka S., Hryshko L.V., Nicoll D.A., Bersohn M.M. and Burke E.P., (1994). Cloning of the NCX2 isoform of the plasma membrane Na+/Ca2+ exchanger. J Biol Chem 269:17434–17439.

Libbey J.E. and Fujinami R.S. (2010). Experimental autoimmune encephalomyelitis as a testing paradigm for adjuvants and vaccines.Vaccine. 29(17):3356-62.

Lipton P. and Lobner D. (1990). Mechanisms of intracellular calcium accumulation in the CA1 region of rat hippocampus during anoxia in vitro. Stroke. 21(11 Suppl):III60-4.

Lo E.H., Dalkara T. and Moskowitz M.A., (2003). Mechanisms, challenges and opportunities in stroke. Nature Rev. 4:399–415.

Lobner D. and Lipton P. (1990). Sigma-ligands and non-competitive NMDA antagonists inhibit glutamate release during cerebral ischemia.Neurosci Lett. 117(1-2):169-74.

Lublin F.D. and Reingold S.C. (1996). Defining the clinical course of multiple sclerosis: results of an international survey. National Multiple Sclerosis Society (USA) Advisory Committee on Clinical Trials of New Agents in Multiple Sclerosis. Neurology . 46:907–11.

LoPachin R.M. Jr and Stys PK. (1995). Elemental composition and water content of rat optic nerve myelinated axons and glial cells: effects of in vitro anoxia and reoxygenation. J Neurosci.15(10):6735-46.

Lytton J., Li X.F., Dong H. and Kraev A. (2002). K+-dependent Na+/Ca2+ exchangers in the brain. Ann N Y Acad Sci. 976:382–393.

Madison D.V., Malenka R.C. and Nicoll R.A. (1991). Mechanisms underlying long-term potentiation of synaptic transmission. Annu Rev Neurosci. 14:379–397.

Martin D.L. and De Luca H.F. (1969). Influence of sodium on calcium transport by the rat small intestine. Am J Physiol. 216:1351–1359.

Martin R., McFarland H.F. and McFarlin D.E. (1992). Immunological aspects of demyelinating diseases.Annu Rev Immunol. 10:153-87.

Matsuda T., Arakawa N., Takuma K., Kishida Y., Kawasaki Y., Sakaue M., Takahashi K., Takahashi T., Suzuki T., Ota T. et al. (2001) SEA0400, a novel and selective inhibitor of the Na+/Ca2+ exchanger, attenuates reperfusion injury in the in vitro and in vivo cerebral ischemic models. J Pharmacol Exp Ther. 298:249–256.

Matsuda T., Takuma K., Nishiguchi E., Hashimoto H., Azuma J. and Baba A., (1996). Involvement of Na+/Ca2+ exchanger in reperfusion-induced delayed cell death of cultured rat astrocytes. Eur J Neurosci. 8:951–958.

Matsuoka S., Nicoll D.A., He Z. and Philipson K.D. (1997). Regulation of cardiac Na+/Ca2+ exchanger by the endogenous XIP region. J Gen Physiol. 109:273–286.

Mattson M.P., Tomaselli K.J. and Rydel R.E. (1993). Calcium-destabilizing and neurodegenerative effects of aggregated beta-amyloid peptide are attenuated by basic FGF. Brain Res. 621:35–39.

McFarland H.F. and Martin R. (2007). Multiple sclerosis: a complicated picture of autoimmunity. Nat Immunol. 8(9):913-9.

McFarlin D.E. and McFarland H.F. Multiple sclerosis (first of two parts). (1982).N Engl J Med. 307(19):1183–1188Michaelis M.L., Johe K. and Kitos T.E. (1984). Age-dependent alterations in synaptic membrane systems for Ca2+ regulation. Mech Ageing Dev. 25:215–225.

Miller S.D., Turley D. M. and Podojil J. R. (2007). Antigen-specific tolerance strategies for the prevention and treatment of autoimmune disease. Nature Reviews Immunology 7, 665-677.

Mitani K., Uchihara T., Tamaru F., Endo K., Tsukagoshi H. (1993). Corticobasal degeneration: clinico-pathological studies on two cases. Rinsho Shinkeigaku. 33(2):155-61.

Mitani A., Yanase H., Sakai K., Wake Y. and Kataoka K. (1993). Origin of intracellular Ca2+ elevation induced by in vitro ischemia-like condition in hippocampal slices.Brain Res. 601(1-2):103-10.

Molinaro P., Cuomo O., Pignataro G., Boscia F., Sirabella R., Pannaccione A., Secondo A., Scorziello A., Adornetto A., Gala R., Viggiano D., Sokolow S., Herchuelz A., Schurmans S., Di Renzo G., Annunziato L., (2008). Targeted disruption of Na+/Ca2+ exchanger 3 (NCX3) gene leads to a worsening of ischemic brain damage. J Neurosci. 28(5):1179-84.

Molinaro P., Viggiano D., Nisticò R., Sirabella R., Secondo A., Boscia F., Pannaccione A., Scorziello A., Mehdawy B., Sokolow S., Herchuelz A., Di Renzo G.F., Annunziato L., (2011). Na+/Ca2+ exchanger (NCX3) knock-out mice display an impairment in hippocampal long-term potentiation and spatial learning and memory. J Neurosci. 31(20):7312-21.

Murray T.J. (2009). The history of multiple sclerosis: the changing frame of the disease over the centuries. J Neurol Sci. 277 Suppl 1:S3-8.

Murray T.J.(2009). Robert Carswell: the first illustrator of MS. Int MS J. 16(3):98-101.

Nagano T., Kawasaki Y., Baba A., Takemura M. and Matsuda T., (2004). Up-regulation of Na+/Ca2+ exchange activity by interferon-gamma in cultured rat microglia. J Neurochem. 90(4):784-91.

Nagano T., Osakada M., Ago Y., Koyama Y., Baba A., Maeda S., Takemura M. and Matsuda. (2005). SEA0400, a specific inhibitor of the Na+/Ca2+ exchanger, attenuates sodium nitroprusside-induced apoptosis in cultured rat microglia. Br J Pharmacol. 144(5):669-79.

Nave K.A. (2010). Myelination and support of axonal integrity by glia. Nature. 468(7321):244-52.

Newell E.W. and Schlichter L.C. (2007). Reversed Na+/Ca2+ exchange contributes to Ca2+ influx and respiratory burst in microglia. Channels (Austin). 1(5):366-76.

Nicoll D.A., Longoni S. and Philipson K.D. (1990). Molecular cloning and functional expression of the cardiac sarcolemmal Na+/Ca2+ exchanger. Science. 250:562–565.

Nicoll D.A., Quednau B.D., Qui Z., Xia Y.R., Lusis A.J. and Philipson K.D. (1996). Cloning of a third mammalian Na+/Ca2+ exchanger, NCX3. J Biol Chem. 271:24914-24921

Nicoll D.A., Hryshko L.V., Matsuoka S., Frank J.S. and Philipson K.D. (1996a). Mutation of amino acid residues in the putative transmembrane segments of the cardiac sarcolemmal Na+/Ca2+ exchanger. J Biol Chem. 271:13385–13391.

Nicoll D.A., Quednau B.D., Qui Z., Xia Y.R., Lusis A.J. and Philipson K.D. (1996b). Cloning of a third mammalian Na+/Ca2+ exchanger, NCX3. J Biol Chem. 271:24914–24921.

Nicoll D.A., Ottolia M., Lu L., Lu Y. and Philipson K.D. (1999). A new topological model of the cardiac sarcolemmal Na+/Ca2+ exchanger. J Biol Chem. 274:910–917.

Nicoll D.A., Ottolia M. and Philipson K.D. (2002). Toward a topological model of the NCX1 exchanger. Ann N Y Acad Sci. 976:11–18.

Paez P.M., Cheli V.T., Ghiani C.A., Spreuer V., Handley V.W. and Campagnoni A.T., (2012). Golli myelin basic proteins stimulate oligodendrocyte progenitor cell proliferation and differentiation in remyelinating adult mouse brain. Glia. 60(7):1078-93.

Paez P.M., Fulton D., Colwell C.S. and Campagnoni A.T. (2009). Voltage-operated Ca2+ and Na+ channels in the oligodendrocyte lineage. J Neurosci Res. 87(15):3259-66.

Paluzzi S., Alloisio S., Zappettini S., Milanese M., Raiteri L., Nobile M. and Bonanno G., (2007). Adult astroglia is competent for Na+/Ca2+ exchanger-operated exocytotic glutamate release triggered by mild depolarization. J Neurochem. 103:1196-1207

Pannaccione A., Secondo A., Molinaro P., D'Avanzo C., Cantile M., Esposito A., Boscia F., Scorziello A., Sirabella R., Sokolow S., Herchuelz A., Di Renzo G. and Annunziato L. (2012). A new concept: Aβ1-42 generates a hyperfunctional proteolytic NCX3 fragment that delays caspase-12 activation and neuronal death. J Neurosci. 32(31):10609-17.

Papa M., Canitano A., Boscia F., Castaldo P., Sellitti S., Porzig H., Taglialatela M. and Annunziato L. (2003). Differential expression of the Na+/Ca2+ exchanger transcripts and proteins in rat brain regions. J Comp Neurol. 461:31–48.

Parpura V. and Zorec R., (2010). Gliotransmission: Exocytotic release from astrocytes. Brain Res Rev. 63:83-92

Patrikios P., Stadelmann C., Kutzelnigg A., Rauschka H., Schmidbauer M., Laursen H., Sorensen P.S., Bruck W., Lucchinetti C. and Lassmann H., (2006). Remyelination is extensive in a subset of multiple sclerosis patients. Brain. 129:3165–3172.

Pettinelli C.B. and McFarlin D.E. (1981). Adoptive transfer of experimental allergic encephalomyelitis in SJL/J mice after in vitro activation of lymph node cells by myelin basic protein: requirement for Lyt 1+ 2- T lymphocytes. J Immunol. 127(4):1420-3.

Philipson K.D., Longoni S. and Ward R., (1988). Purification of the cardiac Na+/Ca2+ exchange protein. Biochim Biophys Acta. 945:298–306.

Philipson K.D. and Nicoll D.A., (2000). Sodium-calcium exchange: a molecular perspective. Ann Rev Physiol. 62:111–133.

Philipson K.D., Nicoll D.A., Ottolia M., Quednau B.D., Reuter H., John S. and Qiu Z. (2002). The Na+/Ca2+ exchange molecule. Ann N Y Acad Sci. 976:1–10.

Pignataro G., Gala R., Cuomo O., Tortiglione A., Giaccio L., Castaldo P., Sirabella R., Matrone C., Canitano A., Amoroso S. et al. (2004a). Two sodium/calcium exchanger gene products, NCX1 and NCX3, play a major role in the development of permanent focal cerebral ischemia. Stroke. 35:2566–2570.

Pignataro G., Tortiglione A., Scorziello A., Giaccio L., Secondo A., Severino B., Santagada V., Caliendo G., Amoroso S., Di Renzo G.F. et al. (2004b). Evidence for a protective role played by the Na+/Ca2+ exchanger in cerebral ischemia induced by middle cerebral artery occlusion in male rats. Neuropharmacology. 46:439–448.

Pignataro G., Boscia F., Esposito E., Sirabella R., Cuomo O., Vinciguerra A., Di Renzo G., Annunziato L., (2011). NCX1 and NCX3: two new effectors of delayed preconditioning in brain ischemia. Neurobiol Dis. 45(1):616-23.

Poser C.M. (1986) Pathogenesis of multiple sclerosis. A critical reappraisal. Acta Neuropathol. 71 1–10.

Prineas J. (1975). Pathology of the early lesion in multiple sclerosis. Hum Pathol. 6(5):531-54.

Quednau B.D., Nicoll D.A., and Philipson K.D., (1997). Tissue specificity and alternative splicing of the Na+/Ca2+ exchanger isoforms NCX1, NCX2 and NCX3 in rat. Am J Physiol 272:C1250–C1261.

Qiu Z., Nicoll D.A. and Philipson K.D. (2001). Helix packing important regions of the cardiac Na+/Ca2+ exchanger. J Biol Chem. 276:194–199.

Remlinger J. (1905). Accidents paralytiques au cours du traitement antirabique. Ann. Inst. Pasteur. 19:625-46.

Reuter H. and Seitz N. (1968). The dependence of calcium efflux from cardiac muscle on temperature and external ion composition. J Physiol. 195:451–470.

Reynolds R., Dawson M., Papadopoulos D., Polito A., Di Bello I.C., Pham-Dinh D. and Levine J. (2002). The response of NG2-expressing oligodendrocyte progenitors to demyelination in MOG-EAE and MS. J Neurocytol. 31(6-7):523-36.

Rivers T.M. and Stewart F.W. (1928). Virus III encephalitis. J Exp Med. 48(5):603-13.

Rivers T.M., Sprunt D.H. and Berry G.P. (1933). Observations on attemps to produce acute disseminated encephalomyelitis in monkeys. J. Exp. Med. 58:39-53.

Rivers T.M. and Schwentker F.F. (1935). Encephalomyelitis accompanied by myelin destruction experimentally produced in monkeys. J. Exp. Med. 61(5):689-702.

Rojas H., Ramos M., Benaim G., Caputo C. and Di Polo R., (2008). The activity of the Na+/Ca2+ exchanger largely modulates the Ca2+i signal induced by hypo-osmotic stress in rat cerebellar astrocytes. The effect of osmolarity on exchange activity. J Physiol Sci. 58:277-279

Rodgers J.M., Robinson A.P. and Miller S.D. (2013). Strategies for protecting oligodendrocytes and enhancing remyelination in multiple sclerosis. Discov Med. 16(86):53-63.

Rowitch D.H. (2004). Glial specification in the vertebrate neural tube. Nat Rev Neurosci. 5(5):409-19.

Saatman K.E., Graham D.I. and McIntosh T.K. (1998) The neuronal cytoskeleton is at risk after mild and moderate brain injury. J Neurotrauma. 15(12):1047-58.

Sabin A.B. and Wright A.M. (1934). Acute ascending myelitis following a monkey bite, with the isolation of a virus capable of reproducing the disease. J. Exp Med. 59(2):115-36.

Schroder U.H., Breder J., Sabelhaus C.F. and Reymann K.G. (1999). The novel Na+/Ca2+ exchange inhibitor KB-R7943 protects CA1 neurons in rat hippocampal slices against hypoxic/hypoglycemic injury. Neuropharmacology. 38:319–321.

Secondo A., Staiano I.R., Scorziello A., Sirabella R., Boscia F., Adornetto A., Canzoniero L.M., Di Renzo G., Annunziato L. (2007). The Na+/Ca2+ exchanger isoform 3 (NCX3) but not isoform 2 (NCX2) and 1 (NCX1) singly transfected in BHK cells plays a protective role in a model of in vitro hypoxia. Ann N Y Acad Sci. 1099:481-5.

Sergeeva O.A., Amberger B.T., Eriksson K.S., Scherer A. and Haas H.L. (2003). Coordinated expression of 5-HT2C receptors with the NCX1 Na+/Ca2+ exchanger in histaminergic neurones. J Neurochem. 87:657–664.

Sirabella R., Secondo A., Pannaccione A., Scorziello A., Valsecchi V., Adornetto A., Bilo L., Di Renzo G. and Annunziato L. (2009). Anoxia-induced NF-kappaB-dependent upregulation of NCX1 contributes to Ca2+ refilling into endoplasmic reticulum in cortical neurons. Stroke. 40(3):922-9.

Sokolow S., Manto M., Gailly P., Molgo J., Vandebrouck C., Vanderwinden J.M., Herchuelz A. and Schurmans S. (2004). Impaired neuromuscular transmission and skeletal muscle fiber necrosis in mice lacking Na+/Ca2+ exchanger 3. J Clin Investig 113:265–273.

Sokolow S., Luu S.H., Headley A.J., Hanson A.Y., Kim T., Miller C.A., Vinters H.V. and Gylys K.H. High levels of synaptosomal Na+-Ca2+ exchangers (NCX1, NCX2, NCX3) co-localized with amyloid-beta in human cerebral cortex affected by Alzheimer's disease . Cell Calcium. 49(4):208-16.

Sospedra M. and Martin R.,(2005). Immunology of Multiple Sclerosis.Annu Rev Immunol. 23:683-747

Sospedra M. and Martin R., (2005). Antigen-specific therapies in multiple sclerosis. Int Rev Immunol. 24(5-6):393-413.

Stallcup W.B. (2002). The NG2 proteoglycan: past insights and future prospects. J. Neurocytol. 31(6-7):423-35.

Steffensen I., Waxman S.G., Mills L. and Stys P.K. (1997). Immunolocalization of the Na+/Ca2+ exchanger in mammalian myelinated axons. Brain Res. 776(1-2):1-9.

Stromnes I.M. and Goverman J.M. (2006). Active induction of experimental allergic encephalomyelitis. Nat Protoc. 1(4):1810-9.

Stys P.K. and Lopachin R.M. (1998). Mechanisms of calcium and sodium fluxes in anoxic myelinated central nervous system axons. Neuroscience. 82:21–32.

Stys P.K., Ransom B.R., Waxman S.G. and Davis P.K. (1990). Role of extracellular calcium in anoxic injury of mammalian central white matter. Proc Natl Acad Sci USA. 87:4212–4216.

Su-Chun Zhang. (2001). Defining glial cells during CNS development Nature Reviews Neuroscience 2, 840-843.

Tafreshi A.P., Mostafavi H. and Zeynali B. (2005). Induction of experimental allergic encephalomyelitis in C57/BL6 Mice: an animal model for multiple sclerosis. Iran J. Allergy Asthma Immunol. 4(3):113-7.

Taglialatela M., Amoroso S., Di Renzo G.F. and Annunziato L. (1988a). Membrane events and ionic processes involved in dopamine release from tuberoinfundibular neurons. I. Effect of the inhibition of the Na+,K+-adenosine triphosphatase pump by ouabain. J Pharmacol Exp Ther. 246:682–688.

Taglialatela M., Amoroso S., Di Renzo G.F. and Annunziato L. (1988b). Membrane events and ionic processes involved in dopamine release

from tuberoinfundibular neurons. II. Effect of the inhibition of the Na+/Ca2+ exchange by amiloride. J Pharmacol Exp Ther 246:689–694.

Takuma K., Matsuda T., Hashimoto H., Asano S. and Baba A. (1994). Cultured rat astrocytes possess Na+/Ca2+ exchanger. Glia. 12:336-342.

Tallantyre E.C., Bo L., A.I.-Rawashdeh O., Owens T., Polman C.H., Lowe J. and Evangelou N. (2009). Greater loss of axons in primary progressive multiple sclerosis plaques compared to secondary progressive disease. Brain. 132:1190–1199.

Thomson A. (2006). FTY720 in multiple sclerosis: the emerging evidence of its therapeutic value.Core Evid. 1(3):157-67.

Thompson A.J. (2001). Symptomatic management and rehabilitation in multiple sclerosis. J Neurol Neurosurg Psychiatry. 2001;71(Suppl. II):ii22–ii27.

Tong X.H., Ding J.W., Yang J., Liu C.Q., Zhang Y., Li S., Li W.H. and Li L. (2009). The effect of protease-activated receptor 2 on rat apoptotic cardiomyocytes underwent ischemia reperfusion injury. Zhonghua Xin Xue Guan Bing Za Zhi. 37(9):832-6.

Trapp B.D., Peterson J., Ransohoff R.M., Rudick R., Mork S. and Bo L. (1998). Axonal transection in the lesions of multiple sclerosis. N Engl J Med., 338:278–285.

van der Valk P. and De Groot C.J., (2000). Staging of multiple sclerosis (MS) lesions: pathology of the time frame of MS. Neuropathol Appl Neurobiol. 26:2–10

Velasquez M.T., Lew S.Q., von Albertini B., Mishkin G.J. and Bosch J.P.(1997). Control of hypertension is better during hemodialysis than during continuous ambulatory peritoneal dialysis in ESRD patients. Clin Nephrol. 48(6):341-5.

Velasquez L.A., Ojeda S.R. and Croxatto H.B. (1997). Expression of platelet-activating factor receptor in the hamster oviduct: localization to the endosalpinx.J Reprod Fertil. 109(2):349-54.

Velasquez M.T., Striffler J.S., Abraham A.A., Michaelis O.E. 4th, Scalbert E. and Thibault N.(1997). Perindopril ameliorates glomerular and renal tubulointerstitial injury in the SHR/N-corpulent rat.Hypertension. 30(5):1232-7.

Verkhratsky A. and Butt A. (2013). Glial Physiology and Pathophysiology. Wiley-Blackwell.

Verkhratsky A., Rodríguez J.J. and Parpura V. (2012). Neurotransmitters and integration in neuronal-astroglial networks. Neurochem Res. 37 (11):2326-38.

Virchow R. (1858). Cellular pathology. As based upon physiological and pathological histology. Lecture XVI-Atheromatous affection of arteries. Nutr Rev. 47(1):23-5.

Wakimoto K., Kobayashi K., Kuro-O M., Yao A., Iwamoto T., Yanaka N., Kita S., Nishida A., Azuma S., Toyoda Y., Omori K., Imahie H., Oka T., Kudoh S., Kohmoto O., Yazaki Y., Shigekawa M., Imai Y., Nabeshima Y. and Komuro I. (2000). Targeted disruption of Na+/Ca2+ exchanger gene leads to cardiomyocyte apoptosis and defects in heartbeat. J Biol Chem. 275(47):36991-8.

Watanabe M., Toyama Y. and Nishiyama A. (2002). Differentiation of proliferated NG2-positive glial progenitor cells in a remyelinating lesion. J Neurosci Res. 69:826–836.

Weiss J.H., Pike C.J. and Cotman C.W., (1994). Ca2+ channels blockers attenuate beta-amyloid peptide toxicity to cortical neurons in culture. J Neurochem. 62:372–375.

Wolf J.A., Stys P.K., Lusardi T., Meaney D. and Smith D.H., (2001). Traumatic axonal injury induces calcium influx modulated by tetrodotoxin-sensitive sodium channels. J Neurosci. 21:1923–1930.

Wu A., Derrico C.A., Hatem L. and Colvin R.A. (1997). Alzheimer's amyloid-beta peptide inhibits sodium/calcium exchange measured in rat and human brain plasma membrane vesicles. Neuroscience. 80:675–684.

Yaghmai A. and Povlishock J. (1992). Traumatically induced reactive change as visualized through the use of monoclonal antibodies targeted to neurofilament subunits. J Neuropathol Exp Neurol. 51(2):158-76.

Yu X.M., Askalan R., Keil G.J. and Salter M.W. (1997). NMDA channel regulation by channel-associated protein tyrosine kinase Src. Science (Wash DC). 275:674–678.

Zamvil S.S. and Steinman L. (1990). The T lymphocyte in experimental allergic encephalomyelitis. Annu Rev Immunol. 8:579-621.

Zinsser H. and Tang F.F. (1926). Immunological studies with herpes virus with a consideration of the herpes – encephalitis problem. J. Exp Med. 44(1):21-34.

Zorec R., Araque A., Carmignoto G., Haydon P.G., Verkhratsky A. and Parpura V. (2012). Astroglial excitability and gliotransmission: an appraisal of Ca2+ as a signalling route. ASN Neuro. 4 (2).

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