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The increase in Semaphorin 3A levels during neuronal differentiation influences axonal elongation and dendritic architecture in human neural progenitors.

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1. PREFACE

Semaphorins as architects of neural landscape

The intricating coordination of guidance cues, orchestrating the development and maintenance of the nervous system, has captivated researchers for decades. Among the conductors of this symphony, a protein family extensively studied in the realm of neurodevelopment is the Semaphorins (SEMAs). SEMAs have emerged as pivotal architects of nervous system, shaping the intricate neural landscape[1].

SEMAs, initially identified for their role in guiding axons, have been proven to be polyhedric regulators that influence a wide spectrum of biological and cellular processes[2]. SEMAs are ubiquitously expressed across all tissues of the body, thereby exerting diverse functions in various systems, including the nervous, circulatory, and immune systems[2]. Particularly, emerging evidence underscores the significant involvement of SEMAs in cancer[3; 4] (Fig. 1).

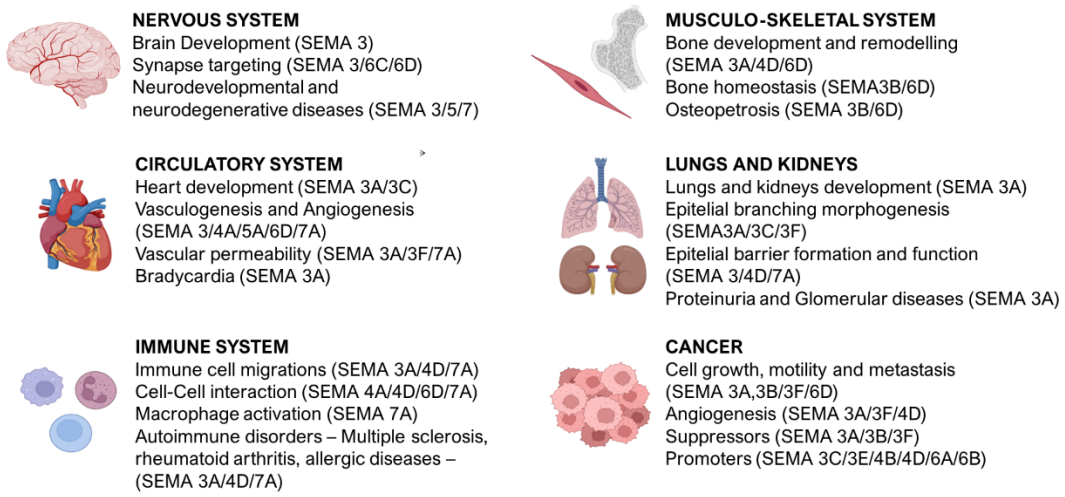


Figure 1. Overview of SEMAs functions in different systems. *SEMAs have been implicated in different functions within the body such as development of brain, heart, bones, lungs, and kidneys. Moreover, SEMAs seem to play a role in neurodevelopmental as well as neurodegenerative diseases and in cancer.*

SEMAs have been best characterized in the nervous system, because of their pivotal roles during the embryonic and early post-natal stages, coordinating the intricate patterns of neuronal growth, guidance, and connectivity[1].

However, recent evidence has shed light on SEMAs also as key regulators of important functions of the adult nervous system. In particular, SEMAs, intricately shape neuronal wiring via:

- **Synapse Formation,** contributing to the regulation of synapse development, maturation, stability, and plasticity[5].
- **Dendritic Morphogenesis,** influencing the growth and branching of dendrites, contributing to the establishment of complex neuronal networks[6].
- **Vasculature Development,** shaping brain blood vessel patterns and ensuring proper blood supply to different brain regions[7].
- **Neurogenesis,** promoting proliferation of neural progenitor cells and differentiation into mature neurons[8].
- **Neural radial migration and laminar distribution,** orientating neurons to distinct layers in the cerebral cortex via cytoskeletal dynamics and directing laminar settling during the early postnatal period[9].
- **Plasticity and Regeneration,** participating in the continuous structural and functional adaptations of the adult brain, impacting on the strength and stability of existing synapses, modulating the robustness of neural connections, and regulating neural regeneration after injury[10].

Indeed, alterations of SEMAs genetics and/or signalling have been implicated in various neurological conditions, encompassing both neurodevelopmental disorders and neurodegenerative diseases including autism[11], epilepsy[12], intellectual disability[13], schizophrenia[14], anxiety and depression[15; 16], Alzheimer's[17] (AD) and Parkinson's disease[17] (PD), amyotrophic lateral sclerosis[18] (ALS), and multiple sclerosis[19] (MS) (Fig. 2).

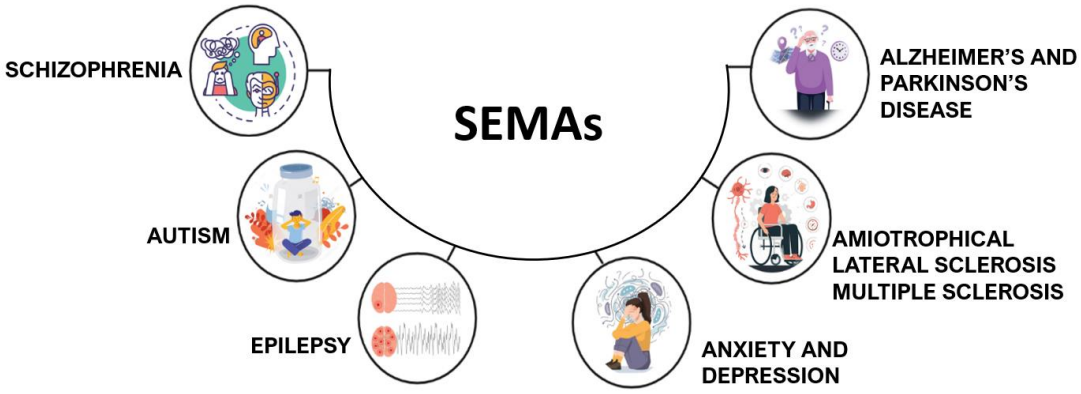


Figure 2. SEMAs implication in neurological diseases. *SEMAs have been associated with various neurological disorders, affecting both neurodevelopment and neurodegeneration.*

A SEMA family member, SEMA 3A has become of particular interest due to its multifaceted role and potential implications in several pathological conditions. As a secreted protein, SEMA 3A is capable of reaching different regions of the body, where it can either regulate vital cellular processes and promote cell survival or initiate harmful events[20].

Originally identified as an axonal chemorepellent, SEMA 3A has emerged as a multifunctional player in various physiological functions including embryonic development, immune modulation, vascularization, and oncogenesis[21; 22].

Extensive research underscored its pivotal involvement in shaping major brain structures, including the brain, spinal cord, and peripheral nerves[23]. Beyond embryogenesis, SEMA 3A continues to exert a regulatory influence on adult nervous system functionality, contributing to axon regeneration and repair[24].

SEMA 3A has also been studied as an immunoregulator that either promotes or suppresses the immune response[25]. This explains the interest in SEMA 3A as a potential therapeutic target for autoimmune diseases[26]. In addition, evidence highlights SEMA 3A emerging role in the vascular system, influencing angiogenesis and promoting vascular remodelling and permeability[27; 28]. Moreover, studies indicated that SEMA 3A also plays a complex role in tumorigenesis and cancer progression, influencing various aspects of cancer biology[22; 29].

Despite it is implicated in all these systems, in this thesis, I will exclusively examine SEMA 3A‘s involvement in developing nervous system, focusing on its physiological role, and emphasizing its relevance in various neurodevelopmental disorders. Moreover, I will discuss SEMA 3A implication in influencing the immune response and its involvement in neuroinflammatory processes.

2. INTRODUCTION

2.1 Semaphorin's classification and receptors

Since the identification of the pioneering members, the Sema family has expanded to encompass 30 proteins, organized into eight classes (SEMA1-8) based on their structural characteristics and functional roles[30](Fig. 3, Table 1). Classes 1 and 2 (Sema 1 and 2) are exclusive to invertebrates, whereas classes 3–7 (SEMA 3-7) are specific to vertebrates. Viruses encode class 8 (Sema 8) members[30]. Notably, transmembrane proteins were identified in Sema 1, SEMA 4, 5, and 6, whereas secreted proteins were characteristic of Sema 2, SEMA 3, and 8. SEMA 7 differs from the others because it is anchored to the cell membrane through a glycosylphosphatidylinositol (GPI) link[30].

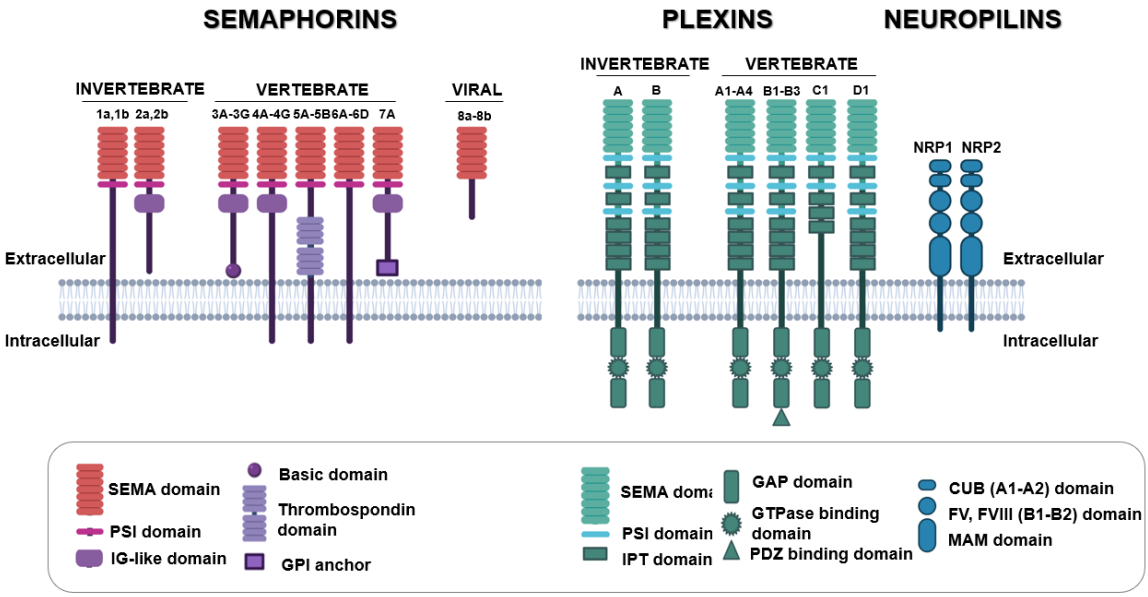


Figure 3. Schematic representation of SEMAs and SEMAs receptors. *Semaphorin family (SEMA, Sema in invertebrates) consist of 8 classes, based on structural criteria. Sema 1(1a-1b) and Sema 2(2a-2b) are found only in invertebrate species, SEMA 3(3A-3G) to SEMA 7 (7A) are found in vertebrates, and SEMA 8 (8a-8b) is reported only in viruses. Sema 2, SEMA 3 and SEMA 8 are secreted proteins, Sema 1, SEMA 4 (4A-4G), SEMA 5 (5A-5B) and SEMA 6 (6A-6D) are transmembrane proteins and SEMA 7 is the only GPI-anchored class. All SEMAs are characterized by a Sema domain and a plexin-sema-integrin (PSI) domain. Sema 2, SEMA 3, SEMA 4 and SEMA 7 share an immunoglobulin (IG)- like domain and SEMA5 exhibit a thrombospondin domain. Plexins (PLXNs, Plexs in invertebrates) are the main SEMA receptors. There are four classes of vertebrate PLXNs (PLXN A-PLXN D), whereas Plex A and Plex B are found only in invertebrate species. PLXNs/Plexs share with SEMA the Sema domain and the PSI domain. The characteristics of all PLXNs/Plexs are the Ig-like,*

plexins, transcription factor (IPT) domain, and the intracellular GTPase activating protein (GAP) domain, which includes one GTPase-binding domain. Remarkably, PLXN Bs show a postsynaptic protein PSD-95/SAP90 (PDZ) domain.

The defining structural characteristic of all SEMAs is a 500-amino acid extracellular domain, at the N-terminal region, known as the SEMA domain[31]. Cryo-EM studies of the SEMA domains revealed a consistent 7-blade beta propeller fold structure across all characterized SEMAs. These structural insights demonstrate that the SEMA domain facilitates homophilic dimerization among SEMAs, consistent with functional studies that emphasize the significance of dimerization in SEMAs activation and function[32].

In addition, a cysteine-rich residue, named plexin-semaphorin-integrin (PSI) domain, which is homologous to the beta chain of integrins and is also found in plexin (PLXNs) family members, is observed at the C-terminus of the SEMA domain. SEMA 2, 3, 4, and 7 also show an immunoglobulin (Ig)-like domain, C-terminal to their PSI domain. Other protein domains exist within the SEMA family, including a basic domain in SEMA 3, thrombospondin repeats in SEMA 5, and GPI-linkage domain in SEMA 7.

SEMAs function through the formation of multimeric complexes with PLXN receptors (Fig. 3, Table 1)[33; 34]. PLXNs are single-membrane-spanning proteins of approximately 240 kDa, considered high-affinity receptors for SEMA, and are categorized into four subfamilies: PLXN As (A1–4), PLXN Bs (B1–3), PLXN C1, and PLXN D1[35].

Their architecture consists of three different extracellular domains: the N-terminal SEMA domain, required for SEMA binding, three PSI domains and six immunoglobulin domains[36]. Four transmembrane domains are responsible for anchoring the proteins to the cell membrane[37]. In addition, in the intracellular region, PLXN proteins exhibit different functional domains including the GTPase-activating protein (GAP) domain and the Rho-GTPase binding protein (RBD) domain, which are crucial for the downstream signalling events triggered by PLXN activation[38; 39]. Of note, PLXN B1 and PLXN D1 share a C-terminal PSD95/Dig/ZO-1 (PDZ) binding motif[40].

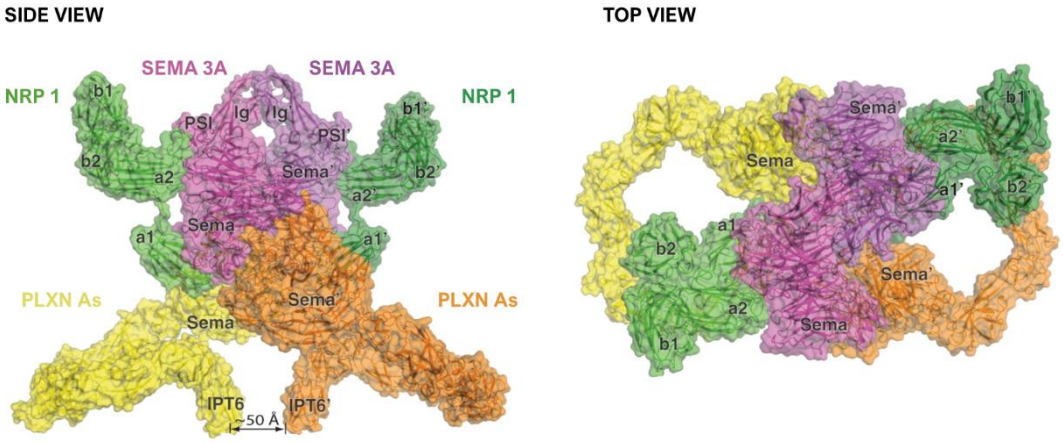
Semaphorin	Receptor Affinity	Semaphorin	Receptor Affinity
Sema 1a-1b	PLXN A	**SEMA 4D**	CD 72 PLXN B1, B2 PLXN C1 PLXN D1
Sema 2a-2b	PLXN B	**SEMA 4E**	Nd
SEMA 3A	NRP 1, PLXN A1- A4 PLXN D1	**SEMA 4F**	PSD-95
SEMA 3B	NRP 1, NRP 2 PLXN A1-A2 PLXN D1	**SEMA 4G**	PLXN B2
SEMA 3C	NRP 1, NRP 2, PLXN A2 PLXN B1 PLXN D1	**SEMA 5A**	PLXN A1, A3 PLXN B3
SEMA 3D	NRP 1PLXN As	**SEMA 5B**	PLXN A1, A3
SEMA 3E	PLXN D1	**SEMA 6A**	PLXN A2, A4
SEMA 3F	NRP 2 PLXN A1- A4	**SEMA 6B**	PLXN A2, A4
SEMA 3G	NRP 2 PLXN As	**SEMA 6C**	PLXN A1
SEMA 4A	Tim 2 PLXN B1, B2 PLXN D1	**SEMA 6D**	PLXN A1
SEMA 4B	Nd	**SEMA 7A**	α1/β1 Integrin PLXN C1
SEMA 4C	PLXN B2	**SEMA 8a-8b**	PLXN C1

Table 1. SEMA classification and receptor affinity*. PLXNs receptors and co-receptors of transmembrane, GPI-anchored, and diffusible SEMA[30; 41; 42; 43; 44]. Tim2=T-cell immunoglobulin mucin receptor 2; Nd=not determined; CD72=Cluster of Differentiation 72; PSD95=post synaptic density protein 95*

Although most of SEMAs bind PLXNs directly, SEMA 3s require the presence of a coreceptor called Neuropilin 1 or 2 (NRP 1, NRP 2) for interaction with PLXNs[45]. SEMA 3E is the only exception, as it binds directly to PLXN D1 in an NRP-independent manner [46]. NRPs are cell surface glycoproteins, with a short 39-amino acid intracellular domain that lacks the intrinsic ability to independently transduce SEMA signals thus serving as a primary ligand binding site, crucial for the formation of the SEMA 3-PLXN signal-transducing active complex[44]. The main feature of NRPs lies in their large extracellular domain, consisting of the N-terminal region referred to as the A1/A2 or CUB domain, followed by two coagulation-factor-homology (FV/FVIII) domains also

called the B1/B2 and a MAM (meprin/A5/μ-phosphatase) domain[47]. Remarkably, cryo-EM studies showed that homo- or heterodimerization of NRPs is an indispensable prerequisite for binding SEMAs and forming functional receptor complex with PLXNs[48] (Fig. 4). Dimerization is also required at the ligand level, where functional SEMA 3 members dimerize via a disulfide bond between the Ig-domain and its basic tail[48] (Fig. 4).

Figure 4. Cryo-EM reconstruction of SEMA 3A-NRP 1-PLXN As complex. *Atomic model*



of the active ligand-receptor complex based on cryo-EM analysis. Colours indicate the boundaries of the single constructs used in the structural analysis. Picture adapted from Lu et al. (2021)[48].

2.2 Semaphorin 3A

SEMA 3A, originally named "collapsin", due to its ability to induce the collapse of the growth cone of dorsal root ganglia (DRG) in chicken embryos[49], was the first SEMA to be described and the most extensively studied up to now. Structurally, SEMA 3A is a secreted protein that exerts both autocrine and paracrine function[50]. It is ubiquitously expressed throughout the body, specifically in the nervous system in both neuronal and non-neuronal cells[11]. Studies analysing SEMA 3A mRNA levels from the 4th week after conception to late adulthood, in 1.340 human tissue samples, collected from developing and adult post-mortem brains from male and female donors of multiple ethnicities, revealed a fluctuation in SEMA 3A expression levels[51] (Fig. 5). In particular, SEMA 3A reaches a peak during the first weeks (4th -10th) after conception, especially in the neocortex, limbic areas, and cerebellum, consistent with its pivotal role in the first stages of embryonic neurodevelopment[51]. However, as its expression persists during adulthood, this suggests the importance of SEMA 3A also in mature nervous system plasticity[17].

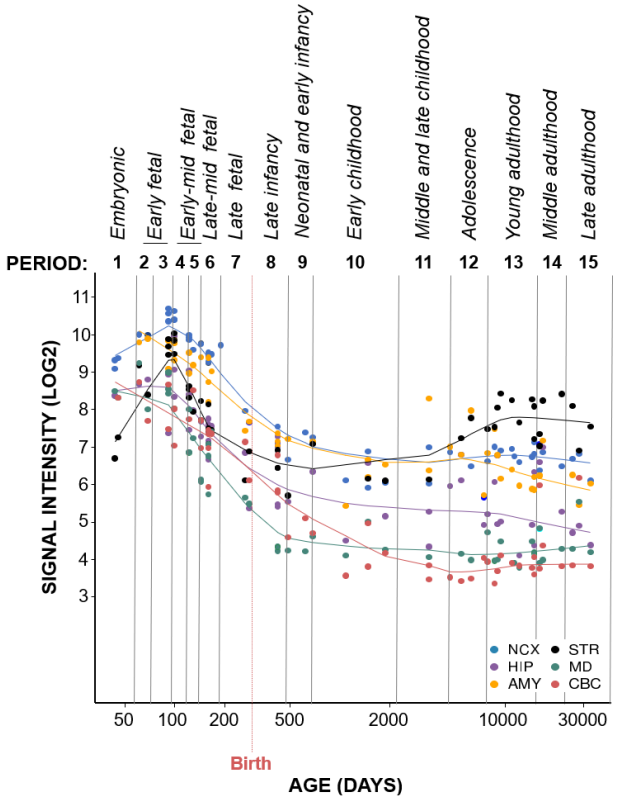


Figure 5. Transcriptomic analysis of SEMA 3A mRNA. *Transcriptomic analysis of SEMA 3A expression. Exon array signal intensity of SEMA 3A in six brain regions. Kang et al. considered a 15-period system, spanning embryonic development to late adulthood. Data and graph were extracted from Human Brain Transcriptomic database[51]. NCX=Neocortex; HP=Hippocampus; AMY=Amygdala; STR=Striatum; MD=Mediodorsal nucleus of the thalamus; CBC=Cerebellar Cortex.*

2.2.1 Semaphorin 3A downstream signalling

SEMA 3A exerts its effects by forming a functional receptor complex with PLXN As and NRPs[20]. Notably, SEMA 3A exhibits a higher affinity for NRP 1 than NRP 2[52], thus making NRP 1 an indispensable component for transducing SEMA 3A signalling[52].

Although the effects of SEMA 3A on cytoskeleton rearrangement are well established, its precise downstream signalling pathways remain elusive. Upon binding to its receptors, SEMA 3A appears to engage different downstream effectors, thereby mediating the diverse functions in axon guidance, dendritic development, and neuronal migration[2; 53]. For instance, different pathways have been described for SEMA 3A signalling (Fig. 6). One includes the Rho GTPase family, which appears to provide a critical link between SEMA 3A and the cytoskeleton (Fig. 6). In particular, Rac-GTPase activity increased following exposure to SEMA 3A. This, facilitates the association between PLXN As and Rnd1, subsequently influencing actin dynamics and cytoskeleton rearrangements[54]. Accordingly, the inhibition of SEMA 3A-dependent growth cone collapse in a Rac dominant-negative mouse model, further highlights the intricate role of Rac-GTPase in mediating these cellular responses[39].

Additionally, SEMA 3A modulates actin and tubulin dynamics by regulating different kinases, including LIM kinase (LIMK), Src tyrosine kinase (TK), and mitogen-activated protein kinase (MAPK)[39](Fig. 6). In particular, LIMK induces phosphorylation and consequent inactivation of the actin-binding protein cofilin, decreasing its ability to promote actin filament turnover and thereby inducing F-actin filament disassembling[55].

SEMA 3A modulates the activity of some kinases belonging to the Src TK family, such as Fes and Fyn[56; 57; 58]. The kinase activity of Fes is instrumental in inducing the collapse of COS-7 cells and growth cones of DRG neurons in response to SEMA 3A[59]. For instance, upon treatment with SEMA 3A, PLXN A1 associates with Fes. Fes, in turn, phosphorylates PLXN A1 and a complex involving collapse response mediator protein 2 (CRMP 2) and CRMP-associated molecule (CRAM)[60].

Considering the role of CRMP 2 in modulating microtubule dynamics, it is plausible that the PLXN A1–Fes–CRMP pathway may contribute to the destabilization of microtubules.

Similarly, PLXN A2 is associated with the TK Fyn[58]. Activated Fyn phosphorylates the serine-threonine cyclin-dependent kinase-5 (CDK5) on Tyr15 residue, promoting the subsequent CRMP2 phosphorylation and cytoskeletal rearrangements leading to the growth cone collapse[57]. Indeed, DRG neurons from Fyn-deficient mice exhibit reduced sensitivity to SEMA 3A-mediated collapse activity[57]. Moreover, following SEMA 3A treatment, evidence suggests that phosphorylation of Tau Ser202 by CDK5 and other kinases counteracts the ability of Tau to bind and stabilize microtubules[17].

Of relevance, Fyn is one of the highest expressed TK in the developing brain[58] and genetic alterations in *FYN* have been found in patients with several neurological conditions, including neurodevelopmental-associated disorders, autism spectrum disorder (ASD) and schizophrenia, highlighting Fyn critical role during neurodevelopment[58].

SEMA 3A also activates extracellular signal-regulated kinase 1 and 2 (ERK 1, 2)/MAPK, which plays a pivotal role in cell proliferation and death[61]. Evidence showed that SEMA 3A, through PLXN A1, impacts growth cone homeostasis by triggering protein synthesis downstream of ERK/MAPK activation[62].

By remodelling the cytoskeleton organization, SEMA 3A also affects cell's adhesion and anchorage to the extracellular matrix (Fig. 6). For instance, SEMA 3A can either decrease cell adhesion by inducing a reduction in integrin activity or by stimulating adhesion-associated proteins such as focal adhesion kinase (FAK) and microtubule-associated protein (MAP) kinase[62; 63]. Notably, SEMA 3A-mediated antagonistic effects on cell adhesion can also be elicited directly, via activation of R-Ras GTPase or indirectly via FARP2 activation, which suppresses R-Ras activity[64].

The ongoing exploration of intracellular molecular partners of SEMA 3A is exemplified by the identification of Molecules that Interact with CAsL (MICAL)[65]. Functioning as a flavoprotein oxidoreductase, MICAL serves as a transducer of SEMA 3A signalling[66]. Indeed, upon exposure to SEMA 3A, MICAL, with its monooxygenase activity, induces the oxidization of actin filaments leading to their severing and decreased polymerization[66]. This molecular cascade ultimately results in the collapse of the growth cones[65].

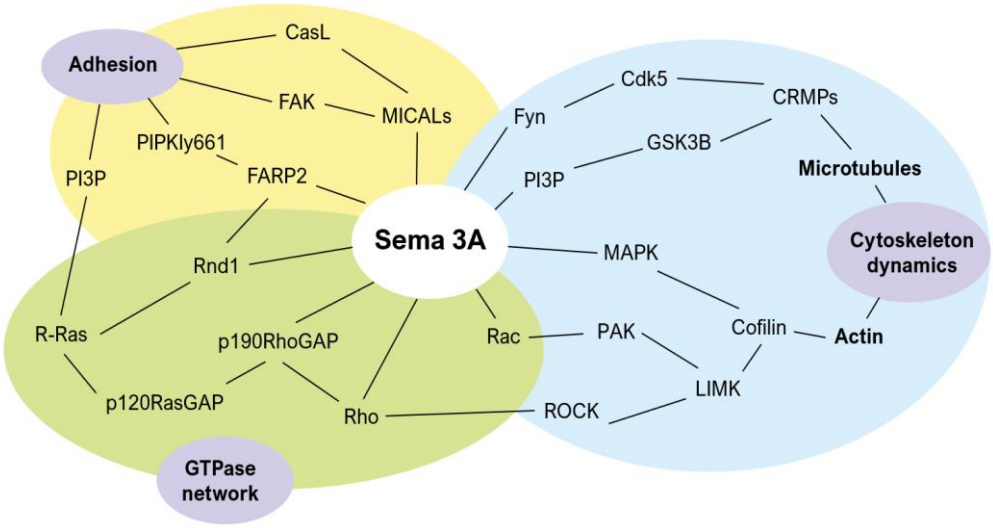


Figure 6. Schematic representation of SEMA 3A signalling*. Cellular responses evoked by SEMA 3A include activation of GTPase network (green oval), remodelling of cytoskeleton through actin and microtubules reorganization (light blue oval) and regulation of cellular adhesion (yellow oval). Image adapted from Jackson et al. (2009)* [67]

2.2.2 Semaphorin 3A expression in the developing nervous system

The establishment of neural circuits requires neurons to differentiate, shape their morphology and form connections with specific cellular targets[9]. These crucial processes involve the formation of dendritic arbours and synaptic contact, initially formed in abundance, and subsequently undergoing refinement with a process called pruning, in order to maintain a lifelong homeostatic balance[1]. Failure to successfully accomplish these developmental milestones can result in disorders that, if not fatal, can lead to long-term disabilities.

Numerous studies reported that SEMA 3A is implicated in the orchestration and regulation of these processes from the very first stages of neuronal development[1; 9; 17; 68].

SEMA 3A plays a pivotal role in influencing neuronal guidance/orientation and migration[8]. Indeed, at sub-nanomolar concentrations, SEMA 3A induces the complete collapse of DRG neurons *in vitro*, underscoring its potent axon repellent activity[49]. Consistently, SEMA 3A mutant mice, exhibit remarkable axon guidance defects and a reduction in dendritic length[69; 70]. Similarly, since embryonic day 10 (E10), SEMA 3A, secreted from the floorplate and the choroid plexus into the cerebrospinal fluid (CSF), regulates the planar orientation of the forebrain and spinal cord progenitors in mice[71].

In the cortex, the complementary attractive and repulsive activity of SEMA 3A and SEMA 3C respectively, confers polarity to pyramidal neurons guiding them from the ventricular zone to their final laminar settling, preserving the proper layer organization[72]. In addition, together with SEMA 3F, the repellent effects of SEMA 3A are crucial for determining the radial migration of GABA-neurons[73] and a specific subset of interneurons[74]. Moreover, SEMA 3A is expressed in the marginal zone and orientates cortical dendrites to the pial surface, promoting their outgrowth and branching[9].

In addition to migration, SEMA 3A appears to play a role also in neuronal maturation.

For instance, SEMA 3A, expressed by cortical inhibitory interneurons[75], can bind to one of the components of peri-neuronal nets, chondroitin sulphate proteoglycans, thus stabilizing and shaping the surrounding matrix and contributing to peri-neuronal net-mediated modulation of neuronal plasticity[21]. Consistently, SEMA 3A in perineuronal nets was found to influence the end of the critical period (time windows of intense brain development and high neuronal plasticity) in the visual cortex, facilitating or restricting plasticity[76].

SEMA 3A also controls synaptic activity and plasticity. In particular, it has been shown that SEMA 3A increases neuronal expression of post-synaptic density protein-95 (PSD-95) and synaptophysin, thus inducing an increase in dendritic spine density and modulating synaptic activity[77]. Accordingly, SEMA 3A and Fyn KO mouse models, exhibit reduced spine density in layer 5 pyramidal neurons[78]. This process of spine modulation seems to be mediated *in vitro* by Cdk5 and *in vivo* by CRMP1[79]. Moreover, SEMA 3A decreases synaptic transmission in the CA1 region of hippocampus [78].

This suggests that SEMA 3A plays a key role in crucial neuronal processes during the initial stages of neurodevelopment. Consequently, it is likely that any disruption in SEMA 3A activity interferes with the physiological progression of brain development, potentially leading to neurodevelopmental disorders.

2.3 Semaphorin 3A in neurodevelopmental disorders

Neurodevelopmental disorders, such as autism spectrum disorder, schizophrenia, and intellectual disability, are early-onset neurological conditions described in the Diagnostic and Statistical Manual for Mental Disorders (DSM-5) (American Psychiatric Association, 2013). These disorders are characterized by abnormalities in cognition, communication, social relationships, behaviour, and motor skills, resulting from disrupted brain development (American Psychiatric Association; 2013).

Recent research suggests that alterations in the expression levels of SEMA 3A and the genetic conditions affecting SEMA 3A, its receptors, or signalling pathways may significantly contribute to the onset of neurodevelopmental disorders by influencing brain connectivity and function.

2.3.1 Autism spectrum disorder

Autism Spectrum Disorder (ASD) is a developmental and neuropsychic condition that emerges early in life and persists throughout the entire individual lifespan[80].

The key features of ASD include impairments in social communication and interaction, repetitive behaviours, and varying levels of intellectual disability [81]. Despite the intricate and multifaceted nature of ASD, genetic factors appear to play a central role in its pathophysiology [82; 83]. Notably, more than 900 genes have been associated with ASD[84; 85; 86] (source: https://www.sfari.org/resource/sfari-gene/), particularly those located on chromosomes 2, 7, 16, 17 [87; 88].

Emerging evidence has highlighted the *SEMA 3A* gene as a potential risk factor for ASD. Indeed, copy number variations (CNVs) in *SEMA 3A* have been reported in 286 patients with ASD[89]. Genetic alterations in SEMA 3A receptors, *NRP* and *PLXN*, have been identified in specific subtypes of ASD, particularly those associated with other comorbidities such as schizophrenia or epilepsy[90; 91; 92]. Consistent with these findings, two heterozygous missense variants, c0.614 G>A (p.Arg205Gln) and c0.4904 G>A (p.Arg1635Gln) in

PLXN A2 were identified in a patient with ASD who also developed epilepsy and attention deficit hyperactivity disorder (ADHD)[93]. Moreover, two single nucleotide polymorphisms (SNPs) in *NRP 2* (rs849578 and rs849563) have been identified in individuals with ASD, suggesting a predisposition conferred by these genes to autism[94].

Additionally, various genes associated with SEMA3A signalling have been identified as mutated in patients with ASD. Thousand-and-one kinase 2 (*TAOK2*), a target of SEMA3A that controls basal dendrite arborization of cortical pyramidal neurons[77], was found to be mutated in an individual with ASD[95].

In another case, a human variant of the large isoform (400kDa) of Ankyrin B (*ANKB*, R2589fs), a SEMA 3A transducer, was reported in an individual diagnosed with ASD[96]. Moreover, heterozygous *de novo* variants of *CRMP1* and *CRMP2*, which are responsible for influencing SEMA 3A-mediated axon guidance[97], have been identified in ASD patients[98].

In addition to genetic effects, ASD is characterized by anomalies in neuronal morphology, connectivity, and organization[99]. For instance, neurons with shorter axons in the amygdala and reduced cell size in the hippocampus have been described in some individuals with ASD[100]. These findings suggest potential anomalies in cerebral connections and dynamics within brain regions associated with emotional and memory processes, both of which are commonly affected in ASD[101; 102]. Similarly, a reduction in axonal extension in the superior temporal gyrus and sulcus has been reported in ASD patients[103; 104]. Such effects may contribute to the lack of facial and eye expressivity as well as diminished responses to auditory and visual stimuli frequently identified in children with ASD traits[105].

Remarkable, SEMA 3A plays a crucial role in the proper formation of major brain structures such as the cerebral cortex, hippocampus, amygdala, olfactory bulb, visual system, and cerebellum [9; 17; 106] .

2.3.2 Schizophrenia

Schizophrenia is a chronic psychiatric disorder characterized by genetic and neurobiological factors that influence early brain development[107]. It manifests as a combination of psychotic symptoms (hallucinations, delusions and disorganized or catatonic behaviour) and motivational and cognitive impairments, including deficits in working memory, executive functioning, and attention[107]. Although symptoms typically emerge in early adulthood, neurobiological alterations relevant to the development of the disorder, appear to be linked to pre- or perinatal complications[108]. For instance, post-mortem examinations of the brains of individuals with schizophrenia have revealed several brain abnormalities, prompting the hypothesis that the disorder may stem from defects in neuronal migration and synaptic connectivity[109; 110].

Of relevance, SEMA 3A expression levels are highly increased in the cerebellum and prefrontal cortex of post-mortem brains of schizophrenic patients compared to those of control subjects[14; 111]. Additionally, genome-wide association studies have identified a correlation between SNPs in the *PLXN A2* receptor and schizophrenia in European, European, American, or Latin-American populations[90]. Intriguingly, this correlation does not extend to Japanese or Chinese patients[112; 113], implying a potential population-specific genetic susceptibility to schizophrenia conferred by *PLXN A2*. Furthermore, Zhao et al. (2018) demonstrated hippocampal neurogenesis defects and social, learning and motor impairments in a knock-out (KO) mouse model of PLXN A2, consistent with the human phenotype of schizophrenia, suggesting a plausible role for SEMA 3A-PLXN A2 signalling in the pathogenesis of the disorder.

Whether alterations in the expression levels of SEMA 3A and/or disruption of the SEMA 3A pathway contribute to the etiogenesis or manifestation of schizophrenia remains a topic of ongoing discussion and requires additional explanation.

2.3.3 Intellectual Disability

Intellectual disability (ID) is a developmental disorder that manifests early in life and affects intellectual functions, such as learning, problem-solving, and judgment. Additionally, it affects adaptive functioning in daily life activities, including communication and independent living (Diagnostic and Statistical Manual of Mental Disorders. 5th ed. American Psychiatric Association; 2013). According to a 2011 meta-analysis, ID is one of the most prevalent disabilities in humans (1% of global prevalence), and one of the most common birth defects[114].

Of note, recent evidence associates genetic variants of SEMA 3A receptors with ID, highlighting the implication of SEMA 3A-PLXN As pathway in the disability onset. In particular, a hemizygous *PLXN A3* variant has been described in a small cohort of young individuals exhibiting ID with co-diagnosed autism, behavioural abnormalities, and epilepsy[13]. Furthermore, both monoallelic and biallelic variants of *PLXN A1* have been documented in ten children, resulting in a neurodevelopmental syndrome characterized predominantly by global developmental delay, craniofacial dysmorphisms, cerebral abnormalities, and visual defects[115]. Lastly, Altuame et al. (2021) proposed *PLXN A2* as a novel gene associated with autosomal recessive ID, mild facial dysmorphisms, and congenital heart disease[116].

2.3.4 Rett Syndrome

Rett Syndrome, is a pervasive developmental disorder attributed to mutations in the transcriptional regulator methyl-CpG binding protein 2 (MeCP2), and considered a prominent cause of mental retardation and developmental regression in girls[117]. Altered expression levels of the SEMA 3A pathway have also been also linked to Rett syndrome[118]. Of relevance, mouse models of the disorder have revealed severe axonal guidance defects in the developing olfactory system associated with altered expression levels in SEMA 3A-NRP 1 pathway[119]. This observation prompted the hypothesis that MeCP2 plays a regulatory role in the expression of SEMA 3A and/or its receptors during

development, thereby contributing to anomalies in neural connectivity under pathological conditions.

2.3.5 Neuro-endocrine syndromes

Consistent with its effects on neuronal guidance, SEMA 3A also plays a role in the development of the neuroendocrine system influencing the migration of gonadotropin-releasing hormone (GnRH) neurons[120; 121]. Hypothalamic GnRH neurons release GnRH triggering the secretion of gonadotropins from the anterior pituitary, thus regulating puberty onset, gametogenesis and oestrous cycling[121]. Defects in GnRH neurons migration during neurodevelopment result in pathological conditions known as hypogonadotropic hypogonadism (HH). Notably, SEMA 3A KO mice exhibit aberrant migration of GnRH neurons, with cells accumulating in the nasal compartment and failing to reach the forebrain[122]. Additionally, mutations in *SEMA 3A, PLXN A1, NRP 1, and NRP 2*, have been identified in several patients with HH [123; 124; 125; 126], highlighting the strong association between SEMA family genes and the development of this neuroendocrine pathology. Of note, we recently proposed SEMA 3A as a novel diagnostic tool to identify a specific subset of patients with decreased ovarian reserve (DOR) who show improved responsiveness to fertilization procedures using GnRH analogues (doi:10.3390/life14030358).

2.4 Semaphorin 3A in immune and neuroinflammatory response

In addition to its established role as a guidance cue, SEMA 3A is recognized as a significant modulator of immune response, influencing its onset or resolution, and plays a pivotal role in immune-mediated inflammatory processes and diseases[127] . Notably, in the human immune system, SEMA 3A is expressed by activated $CD4^+$ and $CD8^+$ T-cells[128; 129], B-cells[130], macrophages[131; 132; 133], dendritic cell (DC)[134; 135] and microglia[136]. Consistent with its function in the nervous system, SEMA 3A regulates immune cell migration, via chemoattraction (as observed for macrophages), or chemorepulsion (as observed for DCs)[127].

SEMA 3A plays a dual role in immune response regulation serving as a suppressor of T-cell function and cytokine production and as an activator of innate immune responses[137] (Fig. 7).

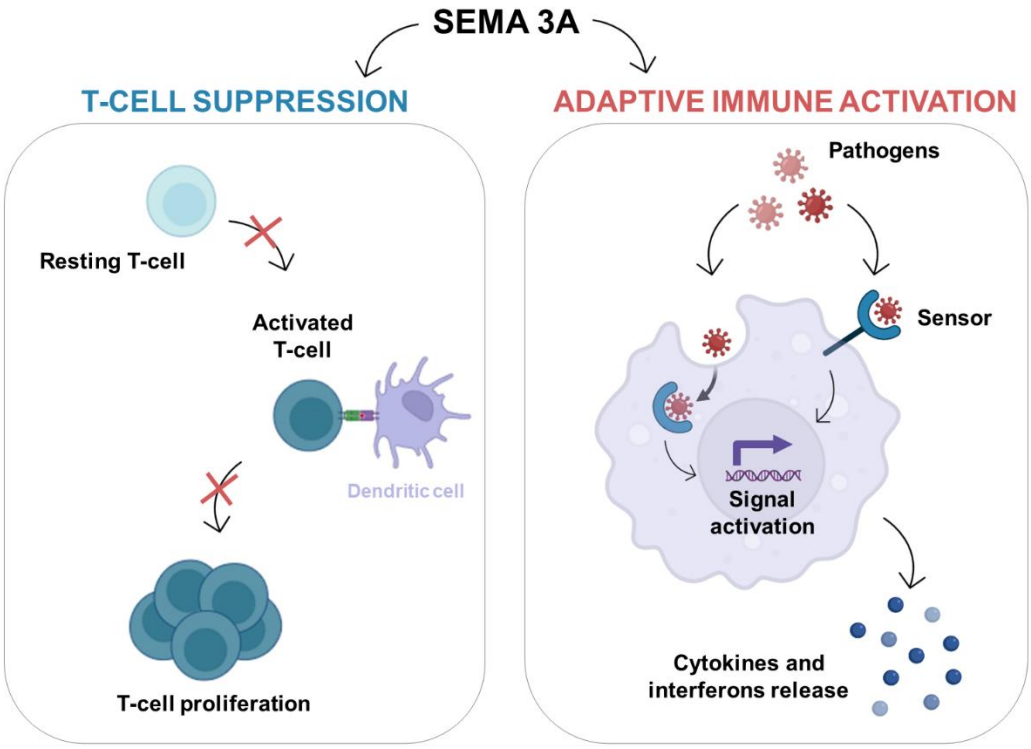


Figure 7. SEMA 3A role in immune system. *SEMA 3A, as immunoregulator factor, has a bifunctional role, suppressing T-cell activity and proliferation and tiggering adaptive immune activation.*

Remarkably, SEMA 3A specifically acts on activated human T-cells expressing NRP 1 and PLXN As receptors, inhibiting their proliferative activity and cytokines secretion (Interleukin (IL) -2, IL-4, IL-10, and interferon (IFN) -γ), when stimulated with anti-CD3/CD28 antibodies[130; 138]. Accordingly, T- cells lacking NRP 1, showed enhanced proliferation after stimulation with an anti-CD3 antibody[127].

The negative regulation of T-cells, driven by SEMA 3A, seems to be associated with the inactivation of ERK1, 2 and the suppression of the Ras/MAPK signalling pathway[130].

Moreover, studies have reported that SEMA 3A induces the activation of proapoptotic processes in both T-cell lines[139] and human macrophages[140], sensitizing cells to Fas (CD95)-mediated apoptosis[139].

In light of its potent immunosuppressive activity, SEMA 3A has been proposed as promising therapeutic target for various immunological diseases including allergic and autoimmune conditions such as allergic rhinitis[141], rheumatoid arthritis[142], systemic lupus erythematosus[143], atopic dermatitis[26; 144], and inflammatory bowel disease[145].

In parallel with the inhibition of T-cells, SEMA 3A was reported to have a stimulatory effect on innate immune response, inducing inflammation[146](Fig. 7). SEMA 3A can modulate neuroinflammatory response, leading to the activation of macrophages and microglia and the release of cytokines and chemokines[127]. During inflammatory events, the local tissue concentration of SEMA 3A may significantly increase, influencing the composition of the inflammatory infiltrate by selectively chemoattracting specific cell types or impeding the migration of others[127]. For instance, tumours overexpressing SEMA 3A exhibit an elevated influx of macrophage[147], along with the exclusion of $CD8^+$ T-cells [132].

Interestingly, SEMA 3A might also be implicated in the activation of the maternal dysregulated immune response after inflammatory triggers or pregnancy complications, leading to cytokine release or anti-brain antibody production, ultimately affecting foetal neurodevelopment (Fig. 8). For instance, SEMA 3A and its receptors (NRP 1 and PLXN A1) are expressed in

the human placenta on both maternal (decidua and uterus) and foetal (umbilical cord) sides[148]. While no evidence of direct correlation has been demonstrated yet between SEMA 3A and vascular and inflammatory conditions during pregnancy, dysregulation in other members of SEMA 3, has been reported. Indeed, elevated SEMA 3C levels are observed at the maternal-foetal placental interface during the first trimester of gestation[149] and SEMA 3F, SEMA 3B and NRP 2 are upregulated in the placenta of women with preeclampsia[150; 151; 152; 153; 154]. This suggests the potential implication of SEMA family and likely SEMA 3A, in influencing communication between maternal and foetal vascularization and controlling the maternal immunological response during pregnancy (Fig. 8).

Noteworthy, anti-brain antibodies, targeting two SEMA 3A transducers, CRMP1 and CRMP2, have been detected in the serum and plasma of pregnant mothers, whose children developed ASD[155; 156; 157; 158.] Anti-brain antibodies are maternal pathogenic antibodies that cross the placenta and reach the foetal circulation, finally targeting foetal brain antigens. Maternal anti-brain antibodies that target SEMA 3A signalling-associated proteins such as CRMP1 or CRMP2, might dysregulate the expression and distribution of these proteins in the developing nervous system, thus resulting in abnormalities in axon guidance, cortical migration, and dendritic projection, likely influencing ASD aetiopathogenesis[159] (Fig. 8).

Microglia, the resident immune cells of the brain[160,] are believed to play a significant role in triggering and sustaining the inflammatory response. Microglia are implicated in several developmental processes, both prenatally and postnatally, including neurogenesis, synaptic pruning, and the organization of functional neural circuits[161]. Accordingly, region-specific microglial activation has been found in the post-mortem brains of individuals with neurodevelopmental disorders, highlighting the emerging role of microglial activation in the onset of these disorders[162.]

Interestingly, SEMA 3A has been shown to directly activate microglia and promote microglia switching into the M1-proinflammatory phenotype, or potentiate microglial response to inflammatory events, likely contributing to

neuronal damage and inflammation-mediated processes described in neurodevelopmental disorders[136] (Fig. 8).

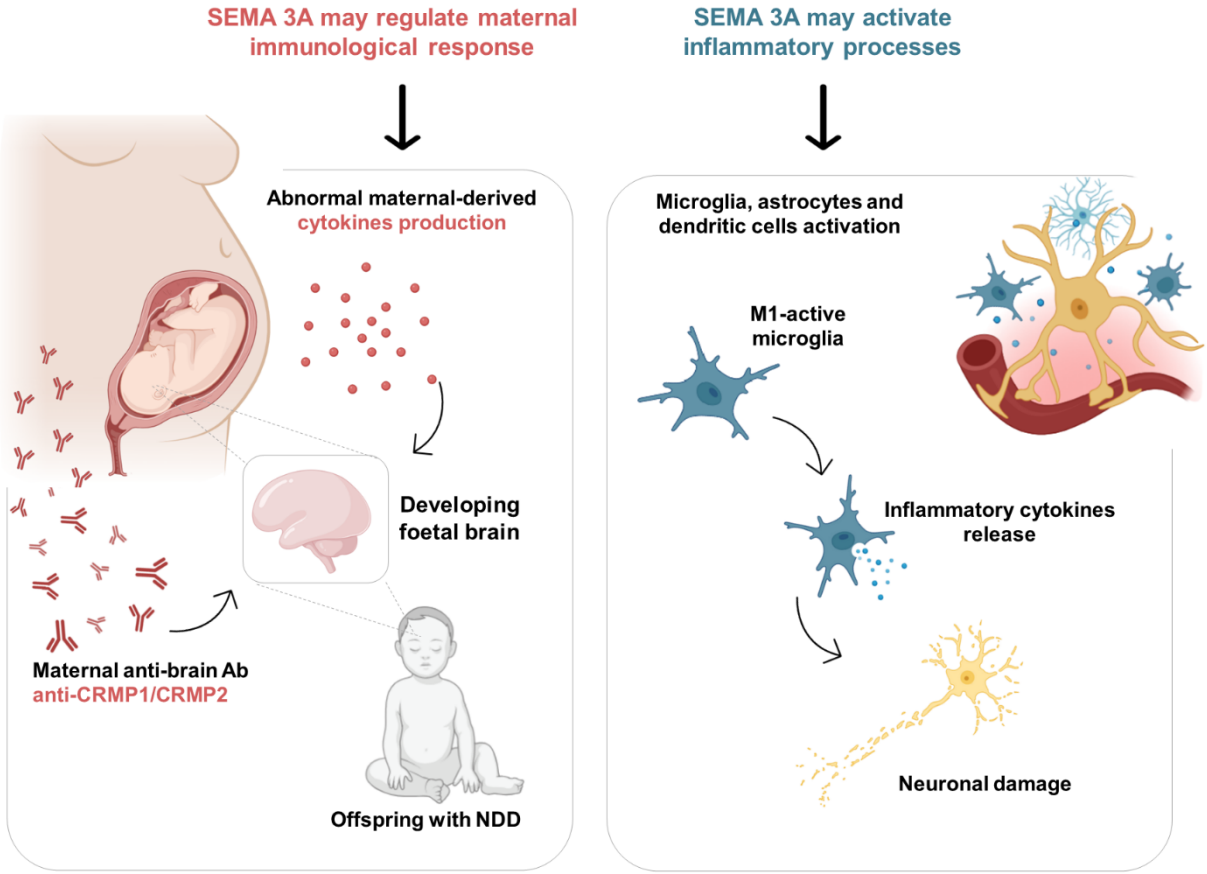


Figure 8. SEMA 3A has potential implications in inflammation-mediated processes that result in susceptibility to neurodevelopmental onset. *(Left) Alterations in SEMA 3A expression during pregnancy can influence the maternal immunological response to inflammatory stimuli. This can lead to the release of inflammatory cytokines or the production of maternal anti-CRMP1,2 autoantibodies, ultimately affecting the developing foetal brain and likely increasing susceptibility to neurodevelopmental disorders. NDD=Neurodevelopmental disorders. (Right) An increase in SEMA 3A promotes microglial switching to the M1-proinflammatory phenotype. This triggers the release of inflammatory cytokines, thereby contributing to neuronal damage.*

3. AIM OF THE THESIS

SEMA 3A has emerged as a pivotal player in orchestrating the development of brain structures that are often impaired in neurodevelopmental disorders, such as ASD, schizophrenia, and ID. Of note, increased levels of SEMA 3A or genetic mutations affecting *SEMA 3A* and/or its downstream pathway(s), have been identified in patients with these disorders. It has been observed that SEMA 3A triggers inflammatory events in both brain cells and circulating blood cells, potentially contributing to neurotoxic processes during foetal development.

Given these findings, this study aimed to investigate the impact of SEMA 3A on neuronal growth and differentiation during the earliest stages of neurodevelopment.

Hence, an *in vitro* model of human neural progenitor's (NP) differentiation was developed. Particularly, the effects of increased SEMA 3A levels on NP were explored using two different experimental strategies. The first approach consisted in the endogenous upregulation of SEMA 3A levels by directly overexpressing SEMA 3A in NP. The second approach focused on exogenously increasing SEMA 3A levels, by exposing NP to media obtained from microglia cells overexpressing SEMA 3A. These experimental paradigms were designed to mimic two pathological conditions described in neurodevelopmental disorders, in which neurons during neurodevelopment may either endogenously overexpress SEMA 3A as a result of genetic mutations or be exposed to external sources of SEMA 3A due to neuroinflammatory processes.

Moreover, the molecular pathway downstream of SEMA 3A signalling was analysed to identify signalling pathways that could potentially be pharmacologically targeted.

4. MATERIALS AND METHODS

4.1 Cell cultures

4.1.1 Human neural progenitors' culture

Human neural progenitors (NP) obtained from cord-blood derived induced pluripotent stem cells (IPSc) of a male newborn (#ax0015) were purchased from Axol Bioscience (Cambridge, UK) and cultured following the supplier's instructions. Briefly, NP were plated in Geltrex (ThermoFisher, Milan, IT)-precoated flasks or coverslips, cultured in Essential media 8 (E8) supplemented with 2% E8 supplement, and differentiated in Neurobasal media supplemented with 2% B27 (ThermoFisher, Milan, IT). As controls for our experiments, some key results were confirmed also in the corresponding female NP cell line #ax0016 (Axol Bioscience, Cambridge, UK).

For DNA transfection experiments, 2 days after plating (days in vitro 2, DIV2) 10 µg/ml of SEMA 3A-GFP (OriGene Technologies Inc., Rockville, MD, USA, #RG213681) or GFP empty vector (OriGene Technologies Inc., Rockville, MD, USA #PS100010) plasmidic DNA were incubated in 1 µl of Lipofectamine Stem Transfection Reagent (Invitrogen, Milan, IT) for 20 min. The mix was then added to NP culture and incubated for 48 h.

For RNA silencing experiments, NP (2,500,000 cell/well, diameter/well 35 mm) at DIV2 were incubated for 48h with a premixed solution of SEMA 3A (Ambion, Milan, IT, #S20284) or NRP 1 (Ambion, Milan, IT, #107,267) or PLXN A2 siRNA (5 pmol, Ambion, Milan, IT, #S10700), 1 µl Lipofectamine Messenger Max mRNA Transfection Reagent (Invitrogen, Milan, IT) and 100 µl Opti-MEM medium (ThermoFisher, Milan, IT).

To set our working conditions, in preliminary experiments, 5 pmol Silencer GAPDH siRNA (Ambion, Milan, IT, #AM4624) and Silencer Negative Control siRNA (Ambion, Milan, IT, #AM4611) were used as the positive and negative control respectively, following supplier's suggestions (data not shown).

In siRNA and DNA co-transfection experiments, NP were incubated with 10 µg/ml SEMA 3A-GFP or GFP-empty vector and 5 pmol siNRP 1 (Ambion,

Milan, IT, #107,267), siSEMA 3A (Ambion, Milan, IT, #S20284) or siPLXN A2 (Ambion, Milan, IT #S10700) in 1 µl Lipofectamine Stem Transfection Reagent (Invitrogen, Milan, IT) and left in culture for 48 h.

4.1.2 Human primary microglia culture

Human foetal brain-derived primary cultures of microglia (#37089–01) were purchased from Celprogen Inc. (Benelux, NL), and cultured in DMEM or Essential medium 8 (ThermoFisher, Milan, IT) supplemented with Essential 8 supplement, according with the necessity of the specific experiment.
For transfection experiments, 10 µg/ml of SEMA 3A plasmidic DNA were incubated with 15 µl of Lipofectamine 2000 (Invitrogen, ThermoFisher, Milan, IT), according to the manufacturer protocols. After 20 min, the mix was transferred to cells and incubated for 48 h. 10 µg/ml of GFP-empty vector was used as transfection positive control following the same procedure.

4.1.3 Neural progenitors exposed to media from microglia overexpressing Semaphorin 3A

Media from SEMA 3A, GFP or non-transfected microglia was collected 48 h after transfection and transferred to NP culture for 1h. In order to minimize events related to changes in growth conditions, microglia and NP were cultured in Essential medium 8 (ThermoFisher, Milan, IT) supplemented with 2% Essential 8 supplement at least 48 h before the experiments.

4.2 Western blotting

To isolate proteins, the cell monolayer was washed once with DPBS and gently scraped in RIPA lysis buffer (ThermoFisher, Milan, IT) supplemented with protease inhibitors cocktail (Sigma-Aldrich, Darmstadt, DE). After 60 min incubation on ice, the homogenates were centrifuged (14,000 rpm, 4 °C, 20 min) and soluble protein samples were stored at -80 °C until use. Protein concentration was determined with the Bradford assay[163]. The standard curve

was generated with serial dilution of bovine serum albumin (BSA) standards. 1µl of protein sample was mixed with 1ml of BioRad Protein Assay Dye Reagent Concentrate (1:5 diluted in distillated water, Biorad Laboratoires, Milan, IT), incubated for 5 min at room temperature and measured the absorbance at 595nm using the Eppendorf BioPhotometer. Then, protein concentration in each sample was calculated using the BSA standard curve. Next, equal amounts (30 µg) of proteins were run on 4–15% Protean-TGX polyacrylamide precast gel (Biorad Laboratoires, Milan, IT) under reducing conditions at constant voltage of 200V for 40 min. Dual colour precision plus protein standard (Biorad Laboratoires, Milan, IT) was used to identify molecular weights. Then, the separated proteins were transferred into PVDF membranes (Abcam, Cambridge, UK) for 1h at 100V/3,5A. Non-specific antibody binding sites were blocked by incubating the PVDF membranes with 5% BSA in Tris-Buffered Saline-Tween (TBS-T, Biorad Laboratoires, Milan, IT) for 1h at room temperature. PVDF membranes were then washed with DPBS and incubated overnight with the appropriate primary antibody. HRP-conjugated anti-mouse or anti-rabbit secondary antibodies (Santa Cruz Biotechnology, Dallas, TX, USA) were used to detect the primary antibody. The protein signal was visualized using chemiluminescent detection Clarity Western ECL Substrate (Biorad Laboratoires, Milan, IT) on a Chemidoc imaging system (Biorad Laboratoires, Milan, IT). Digital quantification of the immunoreactive bands was assessed with ImageLab 6.1.0 software (2020, Bio-Rad Laboratories, Milan, IT). All antibodies used for WB are reported in Table 2.

Antibody	Working Conc..	Reference number
anti-β-actin	1:20000	Sigma-Aldrich, #A3854
anti-Fyn	1:1000	Cell Signaling, #4023
anti-iNOS	1:1000	Proteintech, #18,985–1-AP
anti-p35/CDK5	1:1000	Cell signaling, #2506
anti-pCDK5 Tyr15	1:1000	Cell Signaling, #94,254
anti-pFyn Tyr420	1:1000	Cell Signaling, #2101S

anti-SEMA 3A	1:1000	Invitrogen, #PA5-67,972
anti-TNFα	1:1000	Proteintech #17,590–1-AP
anti-rabbit IgG-HRP	1:5000	Santa Cruz, #sc-2357
anti-mouse mIgGk BP-HRP	1:5000	Santa Cruz, #sc-516102

Table 2. Antibodies used in western blotting experiments. *Conc=concentration*

4.3 Enzyme-linked immunosorbent assay (ELISA)

SEMA 3A protein levels in media from microglia overexpressing SEMA 3A (SEMA 3A media) or GFP (GFP media) or non-transfected (CTRL media) was assessed using ELISA kit (Cusabio, Houston, TX, USA #CSB-E15913h), according to the manufacturer's instructions. Kit detection limit was 0,156 ng/ml, according to the manufacturer's information. Values were normalized on the microglia total cell number.

4.4 Immunofluorescence (IF)

To perform immunofluorescence experiments, approximately 250,000 HNP/slide were plated on Geltrex-precoated glasses coverslips (12mm) and cultured in Neurobasal media supplemented with 2% B27 for 2 days. After two days in vitro (DIV2), NP were either transfected with siRNA/DNA or exposed to microglia conditioned media as described in 4.1.3 section. Next, NP were rinsed once with Dulbecco's Phosphate Buffered Saline (DPBS, ThermoFisher, Milan, IT), fixed in 4% PFA-methanol free solution (ThermoFisher, Milan, IT), and permeabilized with 0.05% Triton X-100 (Bio-Rad Laboratories, Milan, IT) for 3–5 min at room temperature, to ensure free access of the antibody to its antigen. Then, cells were washed twice with DPBS and labelled with the appropriate primary antibody overnight, at 4 °C. Secondary antibodies conjugated with Alexa 488 or Alexa 594 fluorescent fluorochromes were used to detect the primary antibody's signal. Finally, nuclei were stained with 4',6-diamidin-2-fenilindolo (DAPI) and coverslips were mounted upside-down on glass slides using Fluoroshield Mounting

Medium (Abcam, UK). All antibodies, used for IF labelling, are reported in Table 3.

Antibody	*Working Conc.*	*Reference number*
anti-Ankirin G	1:200	Invitrogen, #33–8800
anti-βIII Tubulin	1:1000	Abcam, #ab Ab18207
anti-CD68	1:1000	Proteintech #66,231–2-Ig
anti-CD86	1:1000	Proteintech #13,395–1-AP
anti-IBA1	1:1000	Proteintech, #66,827–1-Ig
anti-iNOS	1:1000	Proteintech, #18,985–1-AP
anti-MAP-2	1:1000	Invitrogen, #PA517646
anti-Npn 1	1:1000	Abcam, #ab81321
anti-Plexin A2	1:1000	Cell Signaling, #5658
anti-TMEM119	1:1000	Proteintech, #66,948–1-Ig
anti-TNFα	1:1000	Cell Signaling, #3707
antimouse-AlexaFluor488	1:250	Invitrogen, #A-11029
antirabbit-AlexaFluor594	1:250	Invitrogen, #R37117

Table 3. Antibodies used in Immunofluorescence experiments. *Conc=concentration*

4.5 Live-cell imaging

Live-cell imaging was performed at CEINGE Advanced Light Microscopy Facility at University of Naples “Federico II”, using the automated platform Celldiscoverer7 (Zeiss, Oberkochen, Germany), equipped with a heated stage (37 °C and 5% CO2) and an Orca flash 4.0 camera (Hamamatsu). Timelapse video (24 frames at 5 min intervals) of HNP exposed to SEMA 3A, GFP and CTRL media were recorded in phase gradient contrast, using a Plan Apochromat 20x/0.7 objective.

4.6 Image acquisition, processing, and analysis.

Images were acquired using a Zeiss LSM700 AxioObserver laser scanning confocal microscope equipped with a gallium arsenide phosphide

photomultiplier tube (GaAsp-PMT) detector, using a Plan Apochromat 40x/1,3 Oil DIC M27 (Zeiss, Oberkochen, Germany) or EC Plan Neofluar 20x/0,50 M27 (Zeiss, Oberkochen, Germany) or EC Plan Neofluar 10x/0,30 (Zeiss, Oberkochen, Germany) objectives.

Microglia in Fig. 13, D was imaged with a Plan Apochromat 63x/1,40 Oil DIC M27 objective (Zeiss, Oberkochen, Germany), on a LSM 980 confocal microscope (Zeiss, Oberkochen, Germany) equipped with using a GaAsP-PMT detector (Zeiss, Oberkochen, Germany) and controlled by Zen Blue Software (Zeiss, Oberkochen, Germany).

Fluorescence microscopy images were processed and analysed using Fiji, ImageJ2 software (National Institutes of Health, Bethesda, Marlan, USA) or Zen 3.1 Software analysis module (Zeiss, Oberkochen, Germany).

In particular, axonal length measurements and Sholl analysis were assessed using NeuronJ and Neuroanatomy Fiji plugs-in respectively. The architecture of dendritic arbour was analysed using Strahler analysis Fiji plug-in.

In addition, representative neuronal skeleton masks in Figs. 11, B and 14, B were obtained after binarization and skeletonization of raw images using Synapse and Neurites Detector (SynD) software.

Quantitative data of microglia were obtained after acquisition of huge tile regions (at least 64 frames) with Celldiscoverer7 system and measurement of Mean Fluorescence Intensity (MFI) with Zen 3.1 Software analysis module (Zeiss, Oberkochen, Germany). A threshold for the MFI was set for each marker within each experiment and cells with fluorescence levels above the set threshold were considered positive for that specific marker. The number of marker-positive cells, expressed in percentage, was normalized for the total number of DAPI-positive nuclei in the analysed field of view (FOV).

In live imaging recordings, axonal retraction was quantified by manual measuring of the distance between the neuronal soma and the axonal edge, at time 0 and after 60 min or 180 min of HNP exposure to SEMA 3A, GFP or CTRL media, using the Zen 3.1 Software analysis module (Zeiss, Oberkochen, Germany).

4.7 Statistical analysis

All data, derived from independent experiments performed at least three times, are presented as mean ± standard error of the mean (SEM). The appropriate statistical test was selected using GraphPad Prism software version 9.0 for Windows (GraphPad Software, San Diego, CA, USA) and reported in the legend of each figure. A probability of P value <0,05 was used to establish statistical significance.

5. RESULTS

5.1 Semaphorin 3A overexpression promotes neuronal death, 48h after transfection

To investigate how human neural progenitors (NP) sense and react to an intracellular increase of SEMA 3A expression during the initial stages of development, we build up an *in vitro* model of human neural progenitors' differentiation. Accordingly with previous investigations conducted by my research group, we observed that approximately 48h after plating, NP initiate the process of polarization and neurite sprouting[164]. Subsequently, 4-5 days later, a substantial amount of NP begins to express microtubule-associated protein 2 (MAP-2), starting their maturation into neurons. This neuronal population continues to proliferate and undergo further differentiation over the following 7-10 days[164; 165]. Henceforth, I will here distinguish and refer to it as NP or neurons depending on whether the cells have been in culture for two (NP) or four days (neurons).

First, to evaluate whether neurons express SEMA 3A and its receptor NRP 1, we labelled them after four days in culture with antibodies targeting SEMA 3A (green) and NRP 1(red, Fig. 9, A). Immunofluorescence analysis revealed that all the neurons expressed both SEMA 3A and NRP 1 (Fig. 9, A). This observation suggests that, at this differentiation stage, there are no specific neuronal subtypes that exhibit a preference for SEMA 3A expression or are more susceptible to SEMA 3A signalling. Interestingly, SEMA 3A was widely distributed in both the cytosol and along neurites, demonstrating substantial colocalization with NRP 1 (Fig. 9, A).

Subsequently, to replicate the alterations in SEMA 3A expression levels described in some neurodevelopmental disorders, neurons were transfected with GFP-tagged SEMA 3A (SEMA 3A). As controls, we used silenced SEMA 3A (siSEMA 3A) and non-transfected neurons (CTRL, Fig. 9, B).

WB experiments revealed a 50% increase in SEMA 3A expression in transfected neurons (Fig. 9, B). Notably, two discernible bands were detected at approximately the molecular weight of 95 kDa and 130 kDa, corresponding

to native SEMA 3A and transfected GFP-tagged SEMA 3A respectively (Fig. 9, B). Importantly, IF analysis demonstrated a remarkable increase in SEMA 3A staining (green) within neuronal apical dendrites 48 h after SEMA 3A transfection compared to the non-transfected control (Fig. 9, A). Conversely, a 60% reduction in SEMA 3A expression was observed in the siSEMA 3A-transfected neurons (Fig. 9, B).

Next, to assess whether SEMA 3A was secreted under our experimental conditions, we performed WB analysis on the media obtained from both SEMA 3A and CTRL neurons. Of note, a slight yet significant increase in SEMA 3A levels was detected in the media from SEMA 3A neurons when compared to the non-transfected controls (Fig. 9, C).

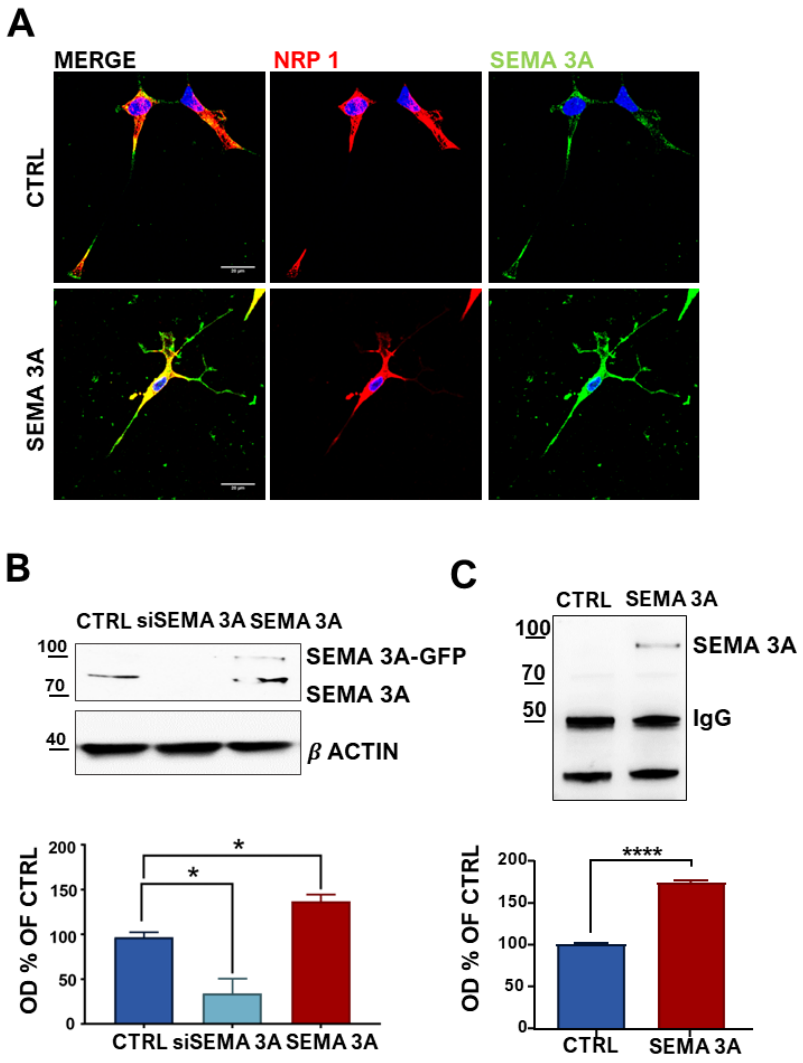


Figure 9. Characterization of CTRL, SEMA 3A, and siSEMA 3A neurons 48 h after transfection. *(A) IF analysis of SEMA 3A (green) and NRP 1 (red) in neurons transfected*

*(SEMA 3A) or non-transfected (CTRL) with SEMA 3A-GFP. Scale bar: 20μm. (B) WB analysis of neurons overexpressing SEMA 3A-GFP (SEMA 3A) or in which SEMA 3A is silenced (siSEMA 3A) 48 h after transfection. Non-transfected neurons were used as control. Optical density (OD) analysis is reported below. (C) Representative WB analysis of media from neurons overexpressing SEMA 3A-GFP (SEMA 3A) or non-transfected CTRL. Data are presented as the mean ± SEM of three independent experiments performed in triplicates. OD analysis is reported below. SEMA 3A levels were normalized to corresponding IgG values (input). Unpaired T-test or one-way ANOVA followed by Tukey's test was used for multiple comparisons. * P<0.05 ****P>0.0001*

In addition, we noted that, the number of cells was significantly reduced 48 h after SEMA 3A transfection, when compared to controls (Fig. 10, A), suggesting that SEMA 3A overexpression influences neuronal survival. Only 15% (± 3.2) of the total DAPI-positive nuclei were properly transfected with SEMA 3A, suggesting that such neurotoxicity is caused by both secreted and intracellular SEMA 3A.

Given that NRP 1 is recognized as essential for mediating SEMA 3A binding to PLXN As receptors, we next evaluated whether NRP 1 silencing could prevent SEMA 3A-mediated neuronal death. To achieve that, we performed in neurons RNA silencing for NRP 1 (siNRP 1) and the positive outcome of this intervention was validated through NRP 1 fluorescence signal (red) quantification, as shown in Fig, 10, B. Relevantly, NRP 1 silencing in SEMA 3A-overexpressing neurons appear to reverse SEMA 3A effects and rescue neuronal survival, reaching a level similar to that observed in CTRL conditions (Fig. 10, A).

Finally, we investigated whether SEMA 3A overexpression triggers the activation of a downstream signalling. Specifically, we assessed the expression levels of Fyn TK, recognized as one of the main downstream signal transducers of SEMA 3A and implicated in the regulation of axonal retraction and dendrite development[166]. Notably, WB analysis revealed an increase in the active Fyn TK, assessed as phosphorylated levels of Fyn Tyr420 residue, 48 h after SEMA 3A transfection. This observation indicated the activation of SEMA 3A downstream signal in neurons overexpressing SEMA 3A (Fig. 10, C).

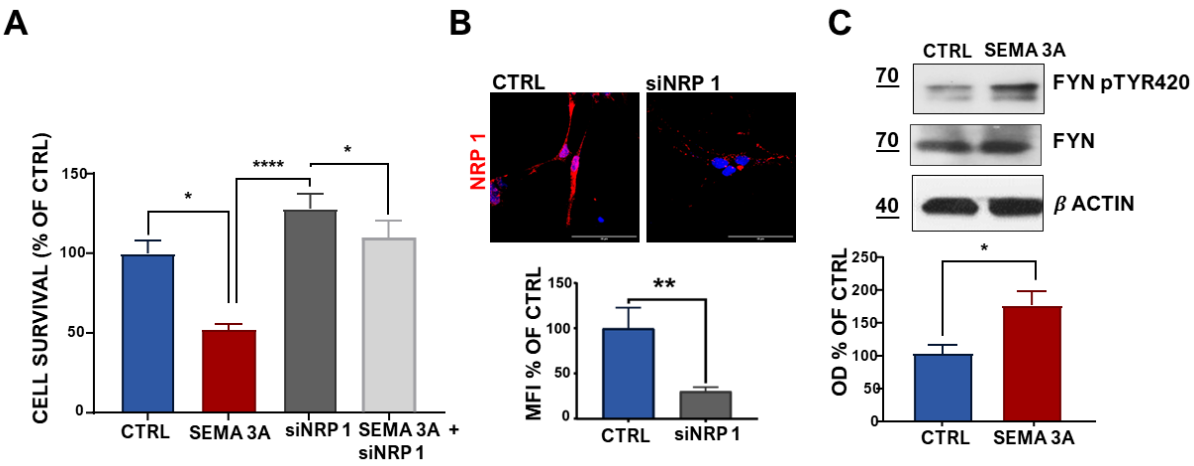


Figure 10. SEMA 3A affects neuronal survival and triggers the activation of downstream signalling 48 h after transfection. *(A) Extent of neuronal survival obtained by counting the number of DAPI positive nuclei before and after SEMA 3A transfection as well as in NRP 1 silencing and CTRL (non-transfected neurons). Data are presented as the mean ± SEM of three independent experiments performed in triplicates. One-way ANOVA followed by Tukey's test was used for multiple comparisons. *P<0.5, ****P<0.0001. (B) Staining quantification of NRP 1 RNA silencing (siNRP 1) expressed as mean fluorescence intensity (MFI). MFI was normalized on the number of DAPI positive nuclei (three slides from three independent experiments). Scale bar: 50μm. 40x objective. Unpaired t-test, **P<0.01 vs CTRL. (C) Representative WB analysis of Fyn pTyr420, Fyn and the correspondent β actin. OD analysis is reported below. Fyn pTyr420 levels were calculated as a ratio of Fyn pTyr420 relative to the corresponding Fyn OD values normalized to β actin (Fyn pTyr420/Fyn). Data are presented as the mean ± SEM of three independent experiments performed in triplicates. One-way ANOVA followed by Tukey's test was used for multiple comparisons. *P<0.5.*

5.2 Semaphorin 3A overexpression induces axonal retraction and increases dendritic arborization in the NP

To visualize neuronal morphology and evaluate the impact of SEMA 3A on neuronal structures, neurons were labelled with Ankyrin G (AnkG, Fig. 11, A, green) as a marker of axon initial segment, and β-III tubulin (β-III Tub, Fig. 11, A, red) or MAP-2 (Fig. 11, D, red) to identify axonal and dendritic filaments respectively.

Morphological analyses, assessed with Image J software, showed that SEMA 3A-overexpressing neurons exhibit a reduction in axonal elongation when compared to CTRL or GFP-transfected neurons (Fig. 11, A, B, C). Interestingly, this event was associated with an increase in dendritic arborization (Fig. 11, D, E, F). Particularly, dendritic branches were significantly increased in the area next to the soma (4-19μm, Fig. 11, E), suggesting that SEMA 3A drives the formation of multiple neurites at the expense of one mature axon. In addition, when we clustered dendritic branches according to Strahler criteria, we observed an increase in I and II orders of branches, exclusively in neurons overexpressing SEMA 3A (Fig. 11, F). This was not observed in CTRL- or GFP-transfected neurons.

Conversely, when SEMA 3A or its coreceptor NRP 1 were silenced, axonal elongation was significantly increased with respect to either CTRL or SEMA 3A-overexpressing neurons (Fig. 11, A, B, C). Notably, when SEMA 3A overexpression was combined with NRP 1 silencing, its effect on axonal retraction was significantly preserved (Fig. 11, A, B, C). This finding indicates that SEMA 3A in this experimental model, requires NRP 1 co-receptor to explicate its function. Moreover, SEMA 3A-siNRP 1 co-transfected neurons, exhibit an atypical dendritic architecture characterized by an increase in the number of branches in the areas distal from the soma (40–50 μm, Fig 11, E), as opposed to that observed after SEMA 3A overexpression.

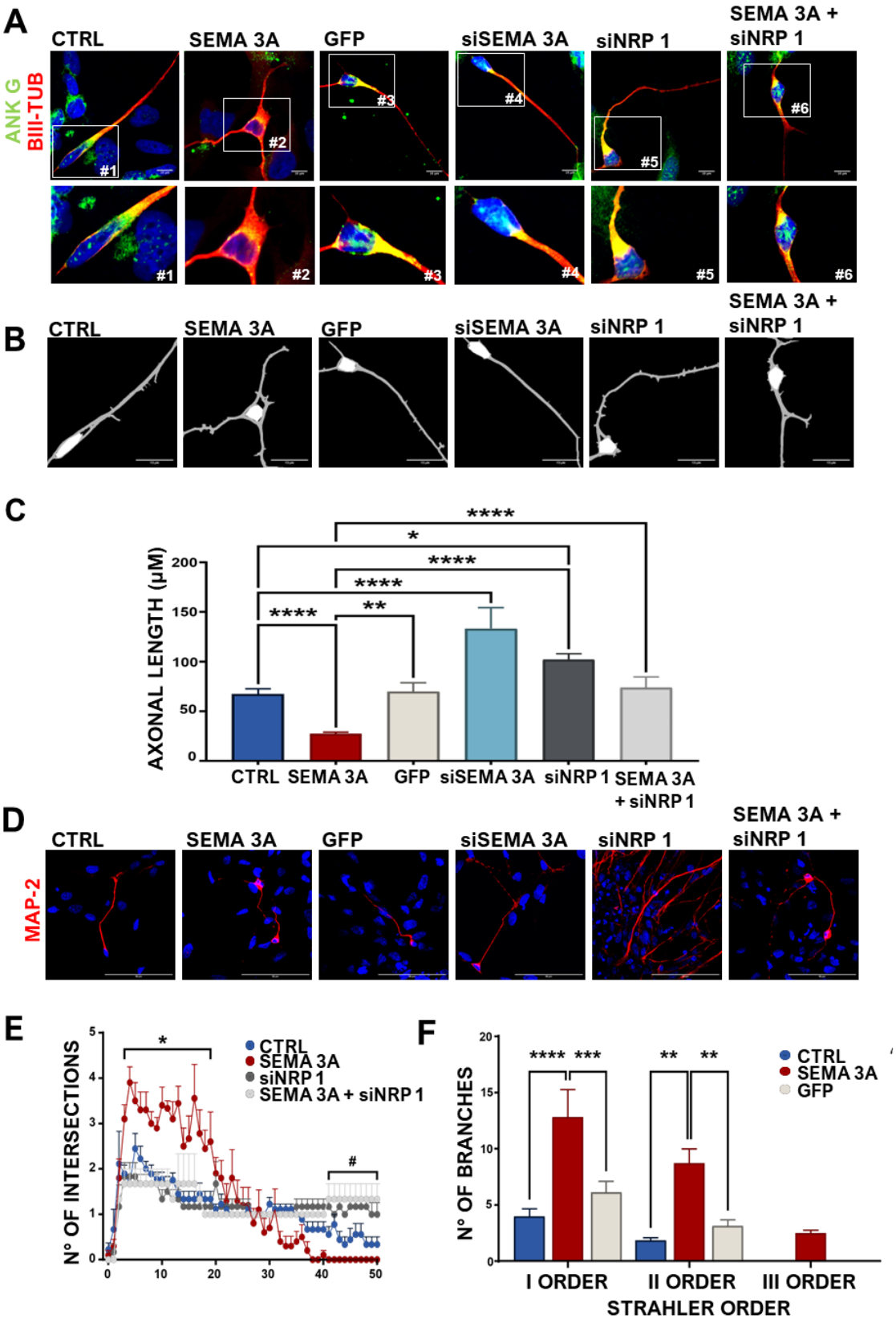


Figure 11. SEMA 3A overexpression induces axonal retraction and increase dendritic arborization in neurons after four days in culture. (*A*) *Representative IF images showing neurons labelled with Ankirin G (green) and β-III tubulin (red) markers of neurons transfected*

*with SEMA 3A (SEMA 3A), SEMA 3A siRNA (siSEMA 3A), NRP 1 siRNA (siNRP 1), and SEMA 3A + NRP 1 siRNA (SEMA 3A + siNRP 1). As controls, we used GFP empty vector (GFP) or non-transfected (CTRL) neurons. Scale bar: 10 μm. 40 × objective. Regions of interest representing the magnification of the axon initial segment, marked with Ankirin G (green) is reported below. The corresponding skeleton masks and the axonal length measurements performed with NeuronJ ImageJ plug-in are reported in (B) and (C) respectively. Data are presented as the mean ± SEM of three independent experiments performed in triplicate (approximately 100 neurons analysed). One-way ANOVA followed by Tukey's test was used for multiple comparisons. *P < 0.05; **P < 0.01; ****P < 0.0001. Statistical significance was set to P < 0,05. (D) IF analysis on neurons stained with MAP-2 dendritic marker. (E) Sholl analysis performed on MAP-2-stained neurons. Data represent the number of dendritic branches at a given distance from the soma (1 μm-radius interval) and are the mean ± SEM of three independent experiments in triplicate. Two-way ANOVA followed by Tukey's test was used for multiple comparisons. *P < 0.05 vs CTRL and #P < 0.05, vs. SEMA 3A + siNRP 1. (F) Strahler analysis using Neuroanatomy plug-in of ImageJ software. At least 10 neurons were analysed in each slide. The experiments were performed in triplicate. Two-way ANOVA followed by Tukey's test was used for multiple comparisons. **P < 0.01; ***P < 0.001; ****P < 0.0001*

5.3 *Microglia overexpressing Semaphorin 3A, release Semaphorin 3A and polarize into the pro-inflammatory phenotype.*

Next, considering that SEMA 3A is a secreted protein, we used a different experimental paradigm consisting of exposing NP to an exogenous source of SEMA 3A. To this end, we used human primary cultures of microglia transfected with SEMA 3A-GFP (SEMA 3A) or GFP empty vector (GFP). Non-transfected microglia were used as controls (CTRL, Fig. 12, A). Therefore, we evaluated the effects of SEMA 3A overexpression on microglial homeostasis. First, after transfection, we found that 17.06% ± 1.1 SEMA 3A and 17.5% ± 2.9 GFP microglia (N = 6; % of the total number of plated cells) were transfected (Fig. 12, A). Additionally, a significant amount of SEMA 3A was released into the media from microglia overexpressing SEMA 3A, as confirmed by ELISA (Fig. 12, B). Moreover, as shown in Fig. 12, C, SEMA 3A overexpression induced a large reduction in the number of microglial cells, when compared to CTRL or GFP.

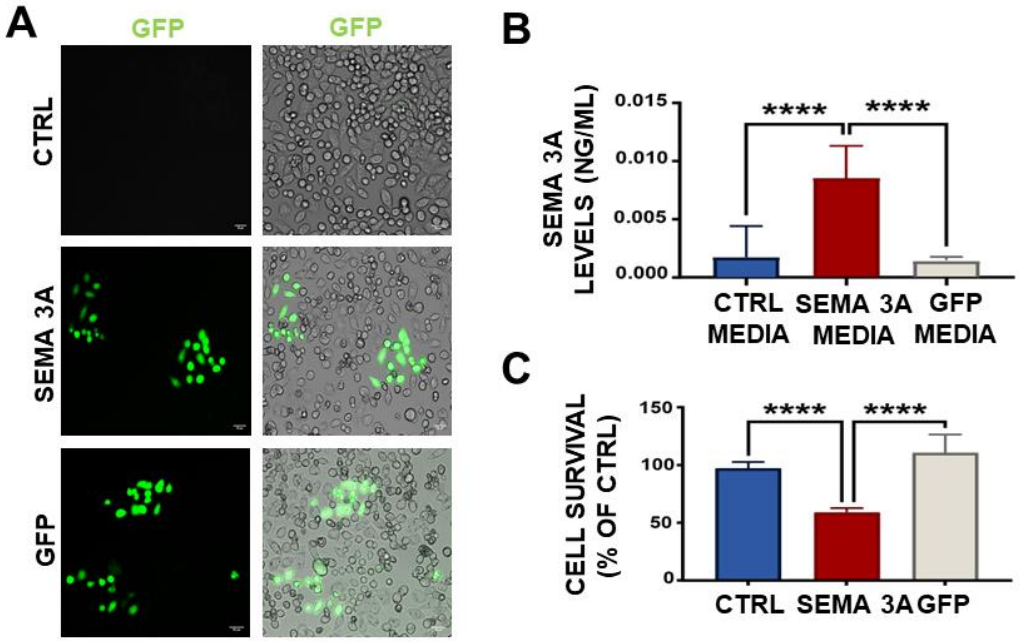


Figure 12. SEMA 3A is expressed and released by SEMA 3A-transfected microglia. *Representative IF images of the percentage of microglia transfected with SEMA 3A-GFP (SEMA 3A) or GFP empty vector (GFP). Non-transfected microglia (CTRL) were used as a control. Scale bar: 55 µm. 10 × objective. (B) ELISA on media from SEMA 3A, GFP, and CTRL microglia 48 h after transfection. SEMA 3A levels are expressed as ng/ml and normalized to*

*the number of alive cells (mean ± SEM from 10 different fields) for each experimental point. ****P < 0.0001 vs SEMA 3A media. (C) The extent of cell survival obtained by counting the number of DAPI-positive nuclei before and after SEMA 3A transfection, as well as in GFP and CTRL microglia. Data are presented as mean ± SEM of three independent experiments in quadruplicate. One-way ANOVA followed by Tukey's test was used for multiple comparisons. ****P < 0.0001 vs SEMA 3A.*

Next, we evaluated whether SEMA 3A overexpression affected microglial activation and polarization 48 h after SEMA 3A or GFP transfection. To this end, microglia were stained with antibodies targeting transmembrane protein 119 (TMEM 119) as a microglia-specific marker or ionized calcium-binding adapter molecule 1 (Iba 1), cluster of differentiation 86 (CD86), and 68 (CD68), which have been reported to be expressed mainly in activated microglia. Finally, tumour necrosis factor α (TNFα) and inducible nitric oxide synthase (iNOS) were used as pro-inflammatory markers to recognise M1 polarized microglia. Therefore, we quantified the mean fluorescence intensity (MFI) for each marker in SEMA 3A- and GFP-transfected microglia and expressed these values with respect to the total cell number and as a percentage of CTRL (Fig. 13 A, B, C). Notably, the number of TMEM 119 positive cells was not significantly different in SEMA 3A-transfected microglia when compared to GFP and CTRL microglia (Fig. 13, B). Conversely, Iba 1, CD86 and CD68 were all increased after SEMA 3A overexpression (Fig. 13, A), suggesting that SEMA 3A activates microglia. Remarkably, the expression levels of iNOS and TNFα were largely increased in microglia overexpressing SEMA 3A when compared to the other experimental points, indicating that SEMA 3A triggers microglial polarization into the M1 pro-inflammatory phenotype (Fig. 13, C, D).

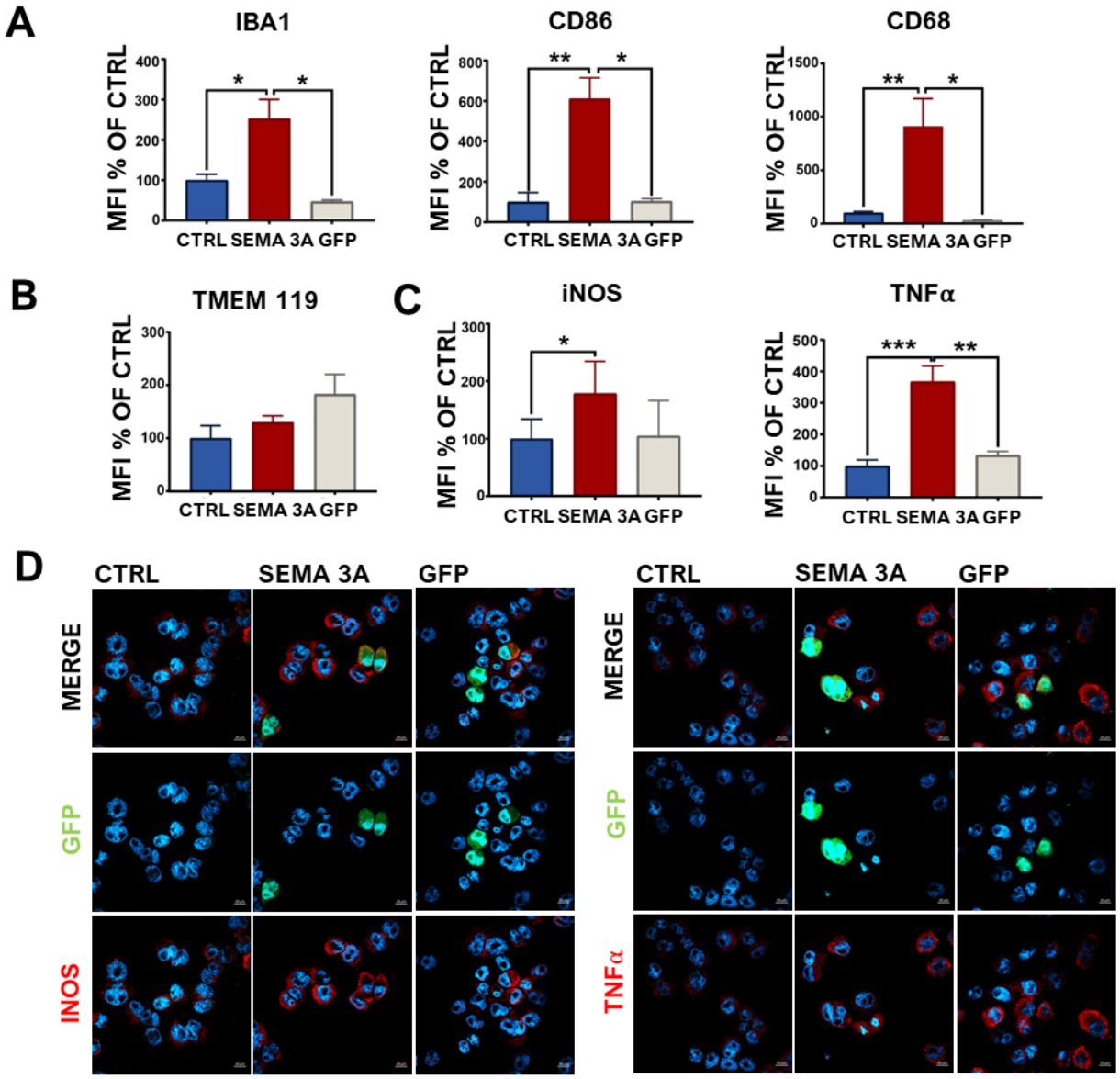


Figure 13. SEMA 3A activates microglia and promotes microglial switching to the M1 proinflammatory phenotype. *Quantitative analysis of Iba1, CD86, CD68 (A), TMEM 119 (B), and iNOS and TNFα (C) expression in SEMA 3A- and GFP-transfected microglia (SEMA 3A, GFP) as well as non-transfected controls (CTRL). MFI was performed on the entire slide using Celldiscoverer7 platform. Values were normalized to the number of DAPI-positive cells for each slide and expressed as a percentage of CTRL. Data are presented as mean ± SEM of three independent experiments performed in quadruplicate. One-way ANOVA followed by Tukey's test was used for multiple comparisons. *P < 0.05; **P < 0.01; ***P < 0.001. Representative images of iNOS (red) or TNFα (red) and SEMA 3A (green) or GFP (green) staining are reported in (D). Scale bar: 10 μm. 63× objective*

5.4 NP exposed to media from microglia overexpressing Semaphorin 3A exhibit morphological changes and activate inflammatory processes

Next, we exposed NP to media from microglia overexpressing SEMA 3A (SEMA 3A media), GFP empty vector (GFP media), or non-transfected microglia (CTRL media) for 60 or 180 min.

First, we evaluated the impact of exogenous SEMA 3A on neuronal morphology by labelling NP with AnkG/β-III tubulin (Fig. 14, A), as axonal markers or MAP-2 (Fig. 15, A) to highlight the dendritic filaments. Interestingly, we found that SEMA 3A media induced a significant reduction in axonal elongation in the NP, as also shown by skeleton masks (Fig. 14, B, C), and an increase in dendritic arborization (Fig. 15, A, B, C). These events occurred within 60 min of exposure and were consistent with our previous data on SEMA 3A-overexpressing neurons. Remarkably, in NP exposed to SEMA 3A media, the number of dendrite branches increased in the areas adjacent to the soma, as shown by Sholl Analysis (Fig. 15, B). In addition, Strahler analysis showed a significant increase in the number of I, II, and III orders of branches in NP after exposure to SEMA 3A (Fig. 15, C), when compared to those exposed to CTRL and GFP media, highlighting that the architecture of dendritic branching increases in complexity in NP exposed to SEMA 3A.

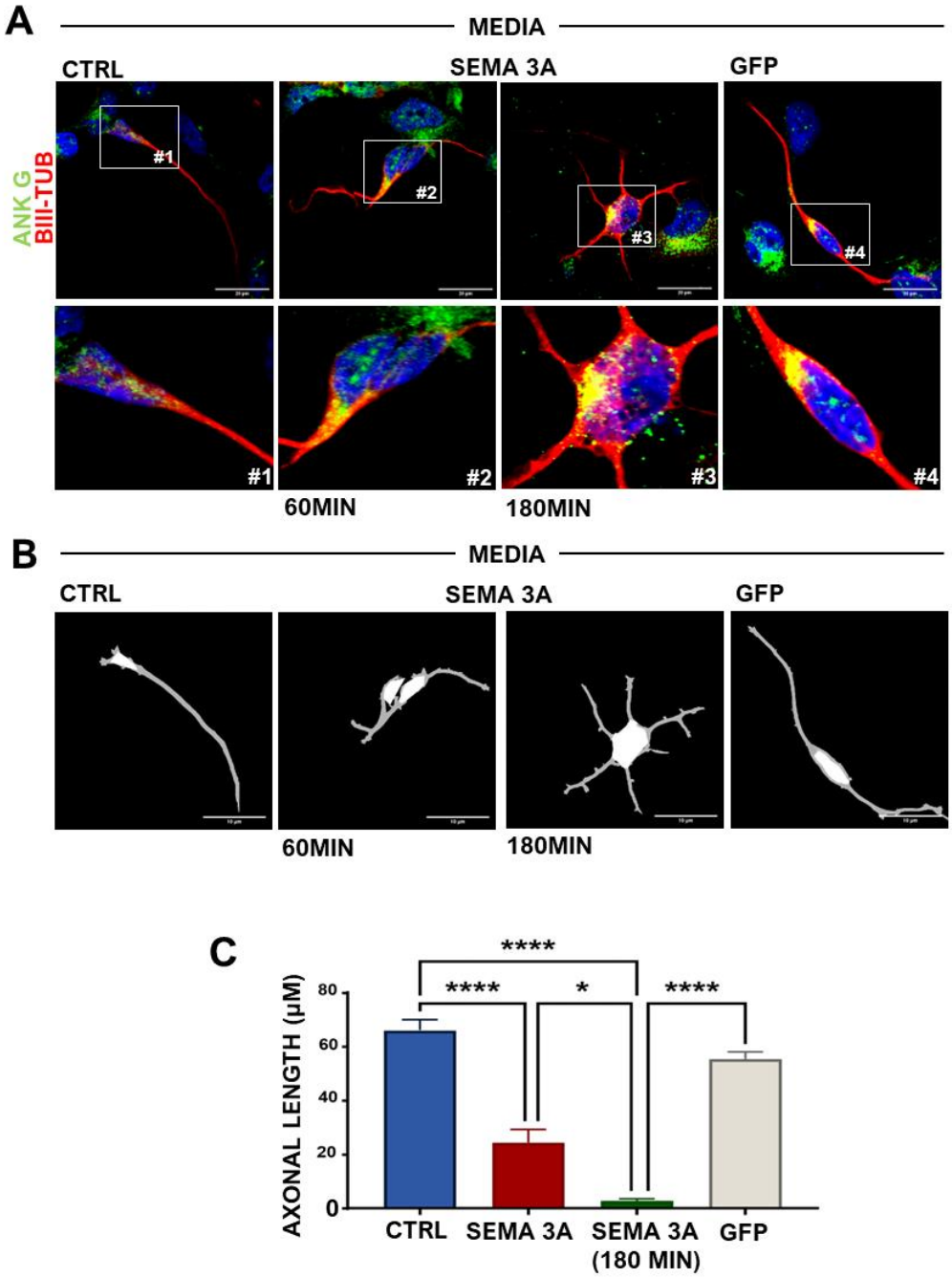


Figure 14. SEMA 3A induces axonal retraction in NP exposed to media from microglia overexpressing SEMA 3A. (*A) Representative Ankyrin G (green) and β-III tubulin (red) images of NP after 60 min and 180 min exposure to media from microglia overexpressing SEMA 3A (SEMA 3A), or GFP empty vector (GFP) or non-transfected microglia (CTRL). Scale bar: 20 µm. Regions of interest showing the magnification of the axon initial segment stained with AnkG (green) is reported below. Scale bar 10 µm. The corresponding skeleton masks and the axonal lengths are shown in (B) and (C) respectively. Analyses were performed using Neuron J plug-in software. Data are presented as the mean ± SEM of four independent experiments performed in duplicate. One-way ANOVA followed by Tukey's test was used for multiple comparisons. *P < 0.05; ****P < 0.0001*

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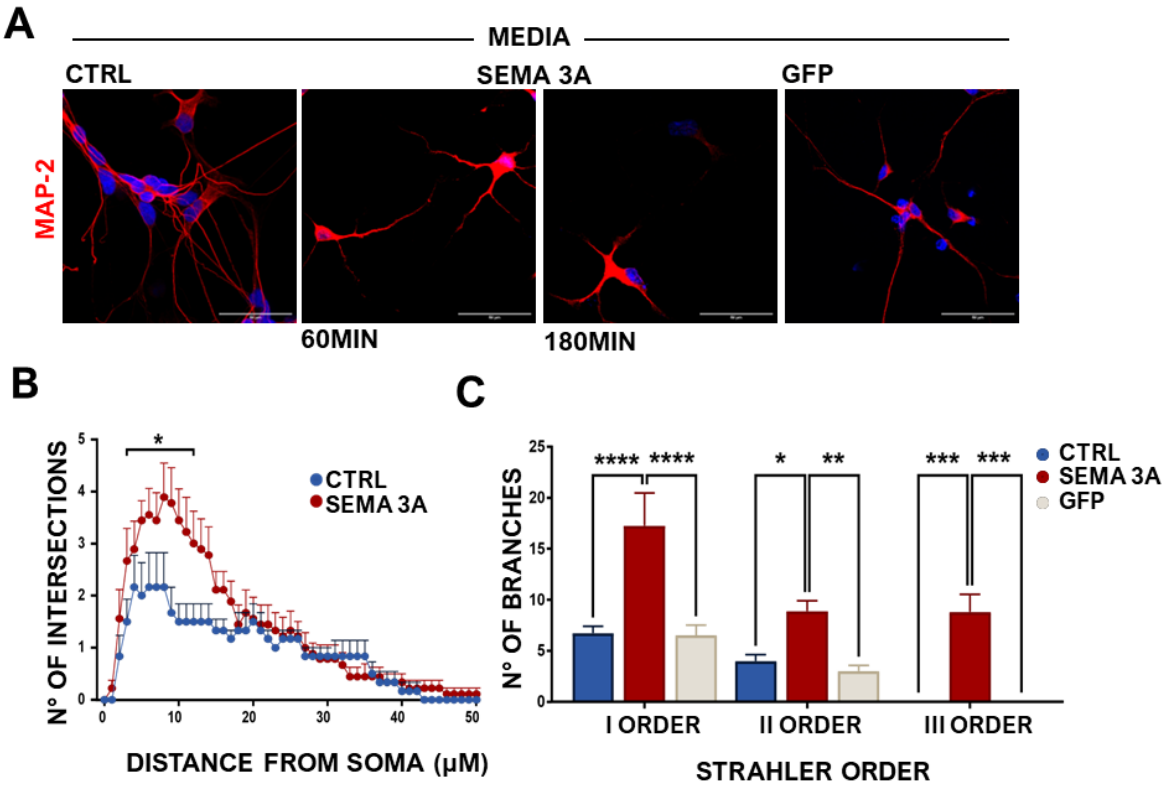

Figure 15. NP exposed to media from microglia overexpressing SEMA 3A exhibit increased dendritic arborization. *(A) Representative MAP-2 staining of NP after 60 min and 180 min exposure to media from microglia overexpressing SEMA 3A (SEMA 3A), or GFP empty vector (GFP) or non-transfected microglia (CTRL). Scale bar 50 µm. (B) Sholl analysis of NP exposed to media from CTRL, SEMA 3A and GFP microglia. Data are presented as the mean ± SEM of three independent experiments performed in duplicate. Two-way ANOVA followed by Tukey's test was used for multiple comparisons. *P < 0.05 vs CTRL. (C) Dendrite clustering analysis according to Strahler's order. Data are presented as the mean ± SEM of three independent experiments performed in duplicate. Two-way ANOVA followed by Tukey's test was used for multiple comparisons. *P < 0.05; **P < 0.01; ***P < 0.001: ****P < 0.0001*

Furthermore, live imaging videos showed that axonal retraction began 30 min after exposure (Fig. 16, A). To quantify this retraction, we measured the distance between the neuronal soma and the axon edge within 60 min after exposure to media from SEMA 3A microglia and found that the NP exposed to SEMA 3A covered a distance approximately doubled with respect to CTRL NP (Fig. 16, C).

Finally, we observed a significant reduction in the number of NP after exposure to SEMA 3A medium for 60 min. Of note, 180 min later, most of the NP were suffering and therefore aggregating in big clusters or dead (Fig. 16,

B, D). This death did not occur in NP exposed to media from GFP or CTRL microglia (Fig. 16, B, D).

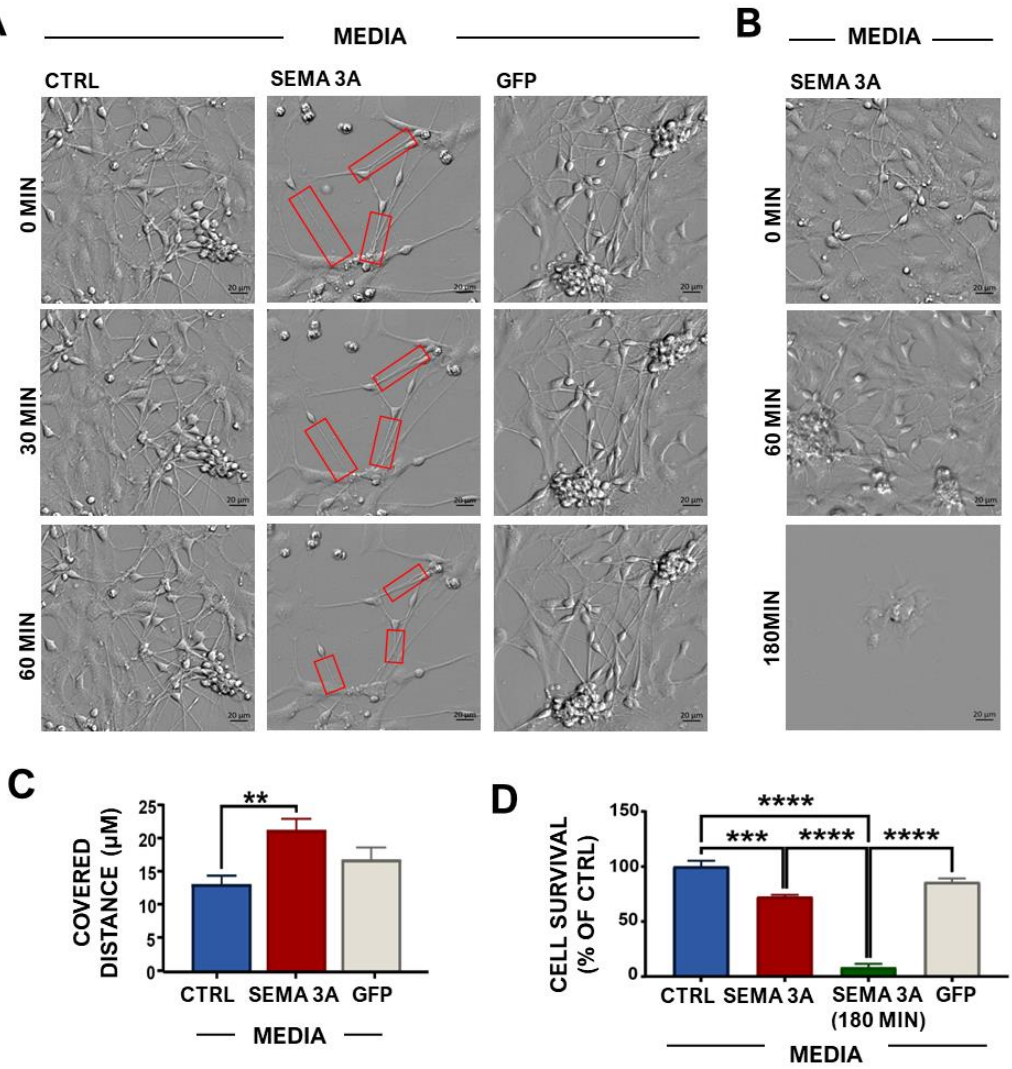

Figure 16. NP exposed to media from microglia overexpressing SEMA 3A cover a larger distance than controls*. (A) Representative contrast microscopy images of axonal retraction extracted from live imaging videos in NP 30 and 60 min after SEMA 3A, CTRL, and GFP media exposure. Scale bar: 20 µm (B) Representative picture of neuronal survival 60 min and 180 min after SEMA 3A media exposure. Scale bar: 20 µm. (C) Covered distance of NP 60 min after SEMA 3A media exposure as well as GFP or control microglia media calculated from live imaging frames. Data are presented as mean ± SEM of three different experiments. One-way ANOVA followed by Tukey's test was used for multiple comparisons. **P < 0.01. Quantitative analysis of DAPI-positive nuclei is shown in (D). Data are presented as the mean ± SEM of three independent experiments performed in triplicate. One-way ANOVA followed by Tukey's test was used for multiple comparisons. ***P < 0.001; ****P < 0.0001.*

To further investigate this aspect and understand whether NP suffered because of the activation of some inflammatory processes, we evaluated the expression levels of two inflammation markers, iNOS and TNFα. Interestingly,

NP exposed to SEMA 3A media exhibited a significant increase in iNOS (Fig. 17, A), and TNF-α (Fig. 17, B) levels when compared to the controls (CTRL media, GFP media), suggesting that SEMA 3A may trigger the activation of inflammatory events that may potentially affect neuronal survival.

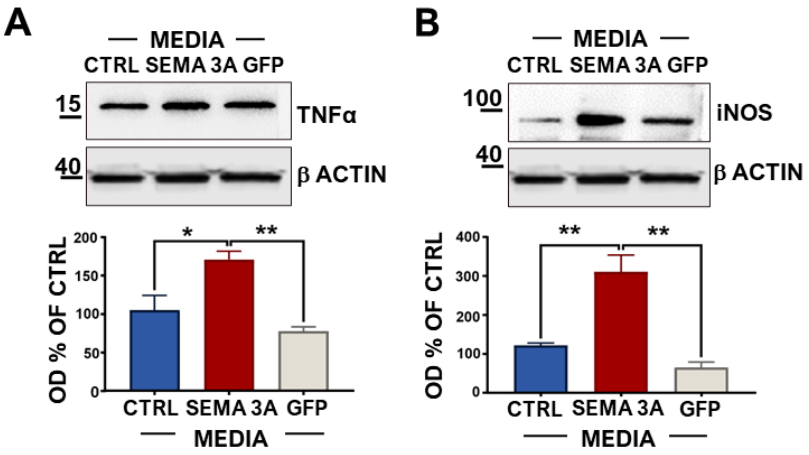

Figure 17. SEMA 3A activates neuroinflammatory processes in NP. *TNFα (A) and iNOS (B) WB analyses on NP after 60 min exposure to SEMA 3A, GFP or CTRL microglia media. Optical density (OD) analysis is reported below. Data are the mean ± SEM of six different experiments and are expressed as % of CTRL. One-way ANOVA followed by Tukey's test was used for multiple comparisons. *P < 0.05; **P < 0.01 vs SEMA 3A*

It's worth noting that a comparable proinflammatory pathway seemed to be triggered in microglia, when exposed to media obtained from neurons overexpressing SEMA 3A (SEMA 3A neuronal media, Fig. 18, A-D). Specifically, there was a notable increase in the iNOS (Fig.18, A, C) and TNFα (Fig. 18, B, D)-positive microglia, which subsequently experienced significant cell death within an hour following exposure to SEMA 3A neuronal media (Fig. 18, E), confirming SEMA 3A proinflammatory and cytotoxic effect.

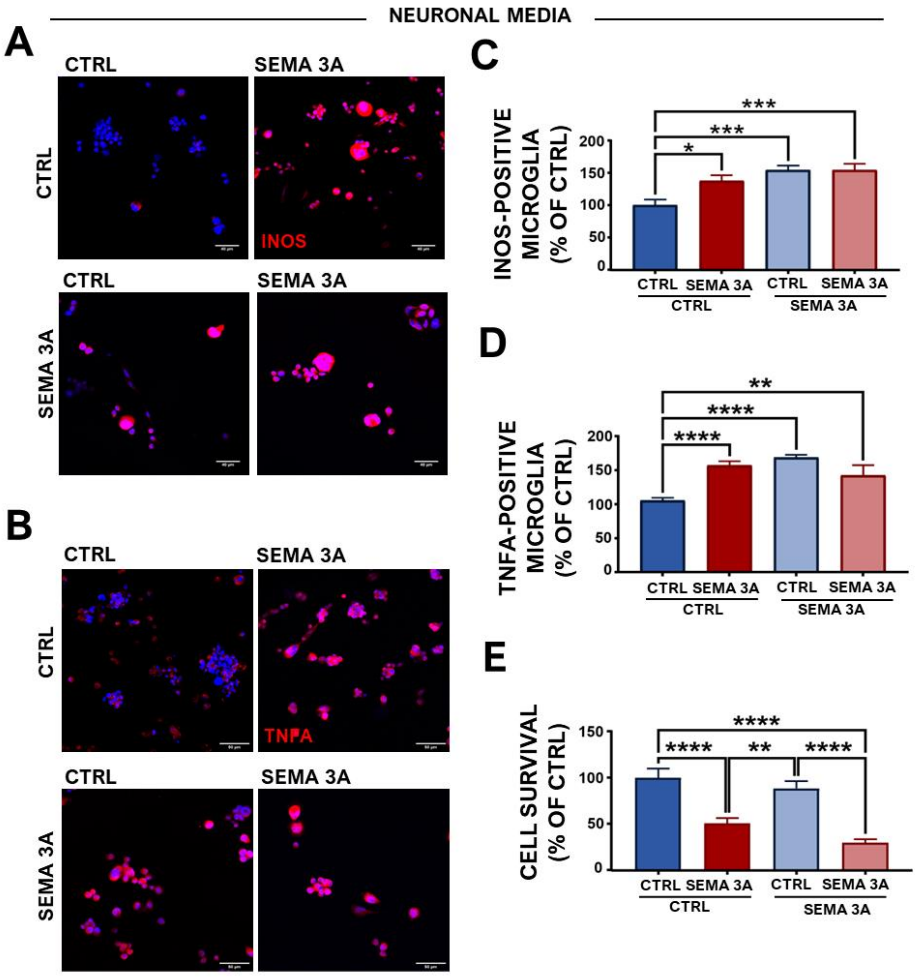

Figure 18. Media from SEMA 3A-overexpressing neurons activate inflammatory processes in microglia after 60min exposure. *iNOS (A) and TNFα (B) representative staining images of microglia cells transfected (SEMA 3A) or not (CTRL) with SEMA 3A-GFP and incubated in media from neurons overexpressing (SEMA 3A neuronal media) or not (CTRL neuronal media) SEMA 3A for 60 min. Scale bar: 40 μm. 20x objective. The number of iNOS and TNFα positive cells is reported in (C) and (D), respectively. One-way ANOVA followed by Tukey's test for multiple comparisons. N=3 *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001. (E) Extent of cell survival obtained by counting the DAPI positive nuclei. Data are the mean ± SEM of five independent experiments and expressed as % of CTRL (non-transfected microglia). One-way ANOVA followed by Tukey's test for multiple comparisons. N=3. **P<0.01; ****P<0.0001.*

5.5 *Semaphorin 3A triggers the activation of PLXN A2-Fyn-CDK5 pathway*

Next, we investigated whether SEMA 3A triggers the activation of a downstream molecular cascade. Although different pathways have been linked to SEMA 3A-induced cytoskeleton reorganization, Fyn TK is considered one of the key transducers of SEMA 3A signalling (Fig. 19, A). Indeed, we performed WB analyses to investigate Fyn activation in NP after exposure to SEMA 3A media. Interestingly, the analyses showed no alterations in the expression levels of Fyn following microglial media exposure (Fig. 19, B). However, a significant increase in phosphorylated levels of Fyn TK on Tyr420 residue was detected in NP exposed to media from microglia overexpressing SEMA 3A (Fig. 19, B), suggesting the involvement of Fyn in mediating SEMA 3A signalling. In addition, Sasaki et al. proposed a mechanism by which Fyn TK induces the phosphorylation of CDK5 on Tyr15[57]. Consistent with these data, the increased levels of Tyr15-phosphorylated CDK5 (Fig. 19, C), and the active fragment p35 (Fig. 19, D), levels were identified upon exposure to SEMA 3A-overexpressing microglia, indicating that SEMA 3A activates the Fyn-CDK5 pathway to mediate its effects.

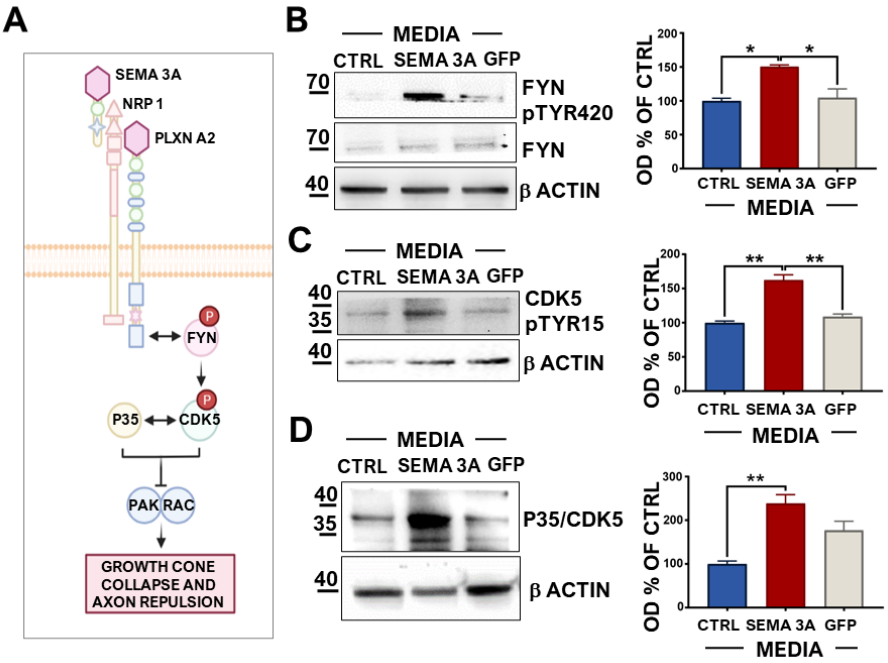

Figure 19. Fyn-CDK5 pathway is activated in NP 60 min after exposure to media from microglia overexpressing SEMA 3A. *(A) Cartoon summarizing the proposed SEMA 3A downstream pathway in NP exposed to SEMA 3A media for 60 min. Representative WB analyses of Fyn pTyr420 and Fyn (B), CDK5 pTyr15 (C), CDK5/p35 (D), and the corresponding β-actin. The OD analysis is shown on the right. The OD values of Fyn, P35/CDK5, and CDK5 pTyr15 were normalized to β-actin and expressed as a percentage of CTRL. Fyn pTyr420 levels were calculated as the ratio of Fyn pTyr420 relative to the corresponding Fyn OD values (Fyn pTyr420/Fyn). Data are presented as the mean ± SEM of three different experiments. One-way ANOVA followed by Tukey's test was used for multiple comparisons. *P < 0.05; **P < 0.01*

As SEMA 3A-Fyn-CDK5 signalling mostly involves the dimerization and activation of the PLXN A2 receptor, we silenced PLXN A2 (siPLXN A2) expression and analysed whether neuronal exposure to SEMA 3A is still able to trigger the Fyn phosphorylation cascade. PLXN A2 silencing was confirmed by WB analysis (Fig. 20, A). Our results showed a reduction in Fyn Tyr420-phosphorylated levels in siPLXN A2 neurons incubated with SEMA 3A media (siPLXN A2 + SEMA 3A media) when compared to neurons in which PLXN A2 was not silenced (CTRL + SEMA 3A media, Fig. 20, B), indicating that the SEMA 3A-mediated axonal retraction signal requires the recruitment of PLXN A2 receptor.

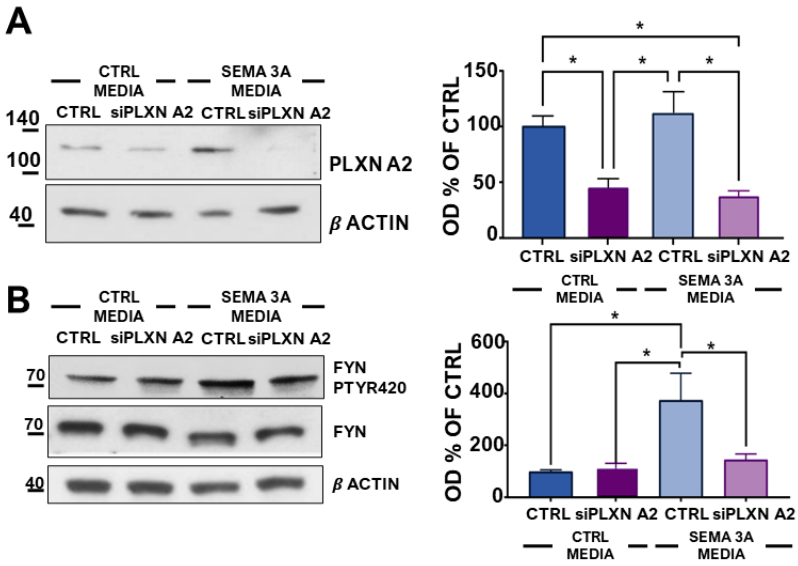

Figure 20. Axonal retraction and Fyn phosphorylation are partially prevented in siPLXN A2 NP exposed to SEMA 3A media*. WB analysis of PLXN A2 (A) or Fyn pTyr420 and Fyn (B), and of the correspondent β actin. The OD analysis is shown on the right. Fyn pTyr420 levels were calculated as a ratio of Fyn pTyr420 relative to the corresponding Fyn OD values normalized to β actin. Data are presented as the mean ± SEM of three different experiments. One-way ANOVA followed by Tukey's test was used for multiple comparisons. *P < 0.05*

6. DISCUSSION

The intricate and highly regulated process of neurodevelopment involves the formation and refinement of neuronal structures, including dendrites, axons, and synapses. Any disruption of these processes can contribute to the manifestation of neurodevelopmental disorders. Neurodevelopmental disorders, such as ASD, schizophrenia, and ID, typically manifest early in life and persist throughout an individual's lifespan. They affect the development and function of the nervous system, affecting various aspects of cognitive, motor, and social functioning[167]. Studies demonstrated the multifactorial aetiology of these disorders, with genetic and environmental factors playing a crucial role[168]. In conditions such as ASD, evidence highlights atypical neuronal morphology, including abnormalities in dendritic arborization, spine density, and synaptic connectivity[99]. These structural changes may underlie the cognitive and behavioural features observed in individuals with ASD. Similarly, in schizophrenia, there are often cortical alterations in dendritic spine density and synaptic connectivity, as well as in the size and complexity of hippocampal neurons[169; 170; 171]. These changes may contribute to deficits in working memory, attention, and emotional dysregulation in patients with schizophrenia.

Accumulating evidence supports the involvement of neuroinflammation in the pathogenesis and progression of neurodevelopmental disorders[172]. For instance, altered levels of inflammatory biomarkers, including reactive oxygen species, chemokines, cytokines, and glial activation, have been reported in many neurodevelopmental disorders, strengthening the hypothesis that neuroinflammation is involved in their pathogenesis[173].

In particular, during pregnancy, environmental insults experienced by the mother are hypothesized to imprint the immune system of the foetus and therefore potentially predispose it to neurodevelopmental disorders[174].

Indeed, foetal exposure to dysregulated maternal immune response, due to infections, autoimmunity, allergies, psychological and social stress, obesity or

pregnancy complications (such as pre-eclampsia), might predispose it to several neuropathology or even death[175; 176; 177; 178; 179; 180; 181; 182].

Interestingly, microglia also seem to play a pivotal role in influencing and regulating foetal immune homeostasis[160]. Disruptions in microglial development and function, coupled with increased density and activation, are crucial factors in neurodevelopmental disorders[183]. Consistently, post-mortem studies in individuals with neurodevelopmental disorders have highlighted region-specific microglial activation, underscoring the emerging implication of microglia in the onset of these disorders[162].

SEMA 3A, is a secreted guidance molecule that assumes a vital role in the development of normal neuronal patterns[184]. Solid experimental evidence suggests that SEMA 3A along with its receptors, is instrumental in influencing the transformation of an immature neural network into a fully functional nervous system[30]. Its significance extends from the initial phases of neurite outgrowth, growth cone navigation, and target innervation to dendritic elaboration, synaptogenesis, experience-dependent remodelling of synaptic connectivity, pruning of exuberant connections, and cell death[1]. Of note, alterations of SEMA 3A expression or genetic mutations linked to SEMA 3A and/or its receptors or pathway-related proteins, have been associated with neurodevelopmental disorders, including ASD, schizophrenia, and ID. In addition, SEMA 3A, acting as an immunoregulator factor, has been reported to influence the immune system either by suppressing T-cell activity or by stimulating the innate immune response, thus promoting inflammation[127]. Accordingly, SEMA 3A can directly activate microglia or potentiate microglial responses to inflammatory stimuli[136]. Moreover, members of the SEMA family have been identified as dysregulated in inflammation-related gestational conditions, such as pre-eclampsia[151; 152].

Giving these considerations, we hypothesized that increased levels of SEMA 3A during the early stages of neuronal development may affect neuronal growth and differentiation. To test this hypothesis, we conducted experiments in which NP were exposed to increased levels of SEMA 3A. This was achieved through direct NP transfection of SEMA 3A or by exposing the cells to media

obtained from microglia overexpressing SEMA 3A. In both experimental paradigms, our findings revealed a significant impact of SEMA 3A on neuronal morphology. Indeed, SEMA 3A acted as both chemorepellent, inhibiting axonal elongation and chemoattractant, stimulating the growth of apical dendrites. Notably, the increase of extracellular SEMA 3A activated neuroinflammatory processes in NP, ultimately compromising neuronal survival. These results, together with the genetic associations of SEMA 3A with neurodevelopmental disorders, shed light on the potential implications of SEMA 3A in neuronal defects and atypical connectivity, as such described in neurodevelopmental disorders.

Additionally, we demonstrated the activation of NRP 1-PLXN A2-Fyn pathway in mediating SEMA 3A signalling. Indeed, SEMA 3A exposure triggers in NP the activation and consequent phosphorylation of Fyn, underscoring its implication as primary transducer of SEMA 3A signal. Interestingly, Sasaki et al. previously proposed a mechanism in which Fyn activation triggers the phosphorylation of CDK5[57]. Accordingly, we found that, when activated, Fyn triggers the subsequent phosphorylation of CDK5, which release the p35 active fragment, activating a cascade of events, finally resulting in cytoskeleton remodelling and growth cone collapse.

Of note, SEMA 3A effects (axonal retraction, aberrant dendritic arborization, and Fyn activation) on NP were prevented when NRP 1 and PLXN A2 receptors were silenced, indicating that SEMA 3A exert its effects by coupling with the NRP1 and PLXN A2 receptor complex. However, the potential contribution of other PLXN As to all the SEMA 3A-mediated processes described cannot be excluded.

Moreover, we found that SEMA 3A promotes the activation of inflammatory processes in both microglia and NP. Particularly, iNOS and TNFα levels increased in microglia after SEMA 3A overexpression, indicating that SEMA 3A promotes microglial activation and polarization into the M1 pro-inflammatory phenotype. In addition, iNOS and TNFα levels were increased in NP 60 min after exposure to SEMA 3A media, indicating that a short inflammatory insult during the very early stages of neuronal development may

promote neuroinflammatory events, thus affecting neuronal differentiation and, in later stages, neuronal functions and connectivity.

Considering these findings and the implications of SEMA 3A in influencing immunological response, it cannot be excluded that SEMA 3A may increase during inflammatory-associated pathological conditions during pregnancy and trigger both maternal and foetal neuroinflammatory processes, which, if occurring during early development, may accentuate foetal vulnerability to neurodevelopmental, immune, and metabolic disorders later in life.

Altogether, these results suggest that an increase in SEMA 3A expression levels serves as a potential trigger of neurodevelopmental defects in human NP and identifies the molecular pathway that mediates SEMA 3A signalling and is responsible for these defects. However, whether SEMA 3A and its effects on neuronal morphology and signalling are involved in the pathogenesis of neurodevelopmental disorders warrant further investigation.

7. CONCLUSIONS AND FUTURE PERSPECTIVES

Altogether, our findings offer novel insights into the role of SEMA 3A in neuronal growth and development within a human model, implementing existing knowledge derived from murine studies. We demonstrated that an increase in SEMA 3A expression levels during NP differentiation exerts significant effects on neuronal morphology, significantly impacting axonal length and dendritic arborization. Additionally, our study reveals that SEMA 3A's effects on NP are contingent upon the specific activation of NRP 1 and PLXN A2 receptors. Moreover, the activation of the SEMA 3A-NRP 1-PLXN A2 complex, initiates a signalling cascade with Fyn TK emerging as the primary transducer of SEMA 3A activity, finally resulting in cytoskeleton rearrangements and cell collapse. Furthermore, our results highlight SEMA 3A implication in initiating inflammatory cytotoxic events, which affect neuronal viability. This occurs through direct alteration of neuronal homeostasis or by instigating SEMA 3A-microglia-mediated neuroinflammatory responses, ultimately exacerbating neuronal death.

In conclusion, this study identifies SEMA3A as a critical regulator of human NP differentiation and elucidates the precise mechanisms underlying SEMA3A-mediated morphological effects on neurons, speculating that a SEMA 3A dysregulation during the first stages of development renders neurons more vulnerable and susceptible to others insults throughout lifespan. These results collectively deepen our understanding of SEMA 3A-mediated neurodevelopmental processes in humans, providing new valuable insights that may significantly impact our comprehension of neurodevelopmental disorders and the design of new therapeutic interventions.

Our future perspectives aim to elucidate the factors responsible for the increase in SEMA 3A levels during neuronal development. One hypothesis posits that SEMA 3A levels may be altered in maternal blood flow in response to hormonal alterations, such as during female reproductive years, or

inflammatory conditions thus potentially affecting conception, embryo implantation, or foetal development.

To delve into this hypothesis, we are currently conducting investigations on two fronts: in collaboration with Prof. Guida from Gynaecology Department at University of Naples "Federico II," we are analysing blood samples obtained from healthy woman, pregnant healthy women, and pregnant women with gestational diseases. The focus is on discerning whether plasma SEMA 3A expression levels fluctuate in these cohorts and whether it is associated with a specific gestational pathological condition.

Simultaneously, we are exploring the potential effects of plasmatic dysregulated levels of SEMA 3A and its correlation with aberrant foetal neurodevelopment. This involves the use of an *in vitro* model of the blood-brain barrier (BBB) and the investigation of the impact of SEMA 3A on BBB permeability. In particular, using super resolution microscopy (SRM) we aim to unravel the effects of SEMA 3A on endothelial cell morphology and function.

Considering this, to improve my microscopy skills and learn SRM techniques, during the last year of my PhD program, I joined the research team led by Prof. Testa at the Royal Institute of Technology, KTH (Stockholm, Sweden). Prof. Testa's group works on developing and applying new super-resolution optical approaches that can help to understand fundamental biological nanoscale processes relevant for health and disease. During this research experience, I acquired knowledge of the physical principles behind SRM, and I maturated practical experience in using different microscopy techniques, as single and multicolour STimulated Emission Depletion (STED) SRM on both fixed and living neuronal samples.

Using this multifaceted (from clinical to molecular) approach, we aim to shed light on the potential connection between the alteration of SEMA 3A expression during pregnancy and its impact on foetal neurodevelopment.

8. DISSEMINATION AND PUBLICATIONS

During my PhD journey, I had the invaluable opportunity to actively participate at different conferences, both on a national and international scale, showcasing my research findings. These platforms served as opportunities to disseminate the key outcomes of my PhD project to a wider academic audience, fostering collaboration and discussion. In particular, the data driven from my PhD investigations were selected for oral or poster presentation at the following conferences:

- **15th Dec 2022, X Neapolitan Brain Group Meeting, Naples, IT (Oral presentation)**

"An increase in Semaphorin 3A compromises axonal elongation and dendritic arborization during human neural progenitors' differentiation."

- **10th-12th Jun 2022, SINS National Meeting of PhD students in Neuroscience, Brescia, IT (Oral presentation)**

"Semaphorin 3A drives axonal growth cone elongation during neuronal differentiation."

- **16th-19th Nov 2022, 41st National Congress of Italian Society Of Pharmacology, Rome, Italy (Poster presentation)**

"Semaphorin 3A overexpression activates neuroinflammatory pathway in human microglia."

- **9th-15th Nov 2022, NEUROSCIENCE 2022, San Diego, CA, USA (Poster presentation)**

"Secreted Semaphorin 3A compromises axonal elongation and dendritic arborization in an in vitro model of human neural progenitors during differentiation."

- **9th-13th Jul 2022, FENS FORUM 2022, Paris, France (Poster presentation)**

"Semaphorin 3A controls axonal growth cone elongation and dendrite's arborization in human neural progenitors."

- **10th-11th Sept 2021, National Congress of the Italian Society for Neuroscience, Naples, IT (Poster presentation)**

"An unbalance in Semaphorin 3A expression and activity alters human neural stem cells morphology and differentiation."

Furthermore, I am honoured that my PhD journey has culminated in the successful publication of two research articles within the realm of neurodevelopment, a focal point of my doctoral project. In particular, the first article, derived from the data presented in this thesis, identifies SEMA 3A as a critical regulator of human NP differentiation and describes the effect of SEMA 3A overexpression on neuronal growth, morphology, and connectivity. The second article, a comprehensive research review, explores the emerging evidence regarding the role of SEMA 3A in regulating neurodevelopmental processes that appear to be dysfunctional in patients with ASD and provides new insights on the potential diagnostic and therapeutic implications of SEMA3A-targeted approaches.

PAPER 1

Ferretti G., Romano A., Sirabella R., Serafini S., Maier TJ, Matrone C.
An increase in Semaphorin 3A biases the axonal direction and induces an aberrant dendritic arborization in an in vitro model of human neural progenitor differentiation.
Cell Biosci. (BMC) 2022 PMID: 36348448

PAPER 2

Matrone C. and **Ferretti G.**
Semaphorin 3A influences neuronal processes that are altered in patients with autism spectrum disorder: Potential diagnostic and therapeutic implications mechanisms.
Neurosci and Biobehav Rev, 2023 PMID: 37524141

Moreover, in tandem with my PhD project mainly focused on neurodevelopment, I actively contributed to additional projects conducted in my research group, as evidenced by the publications reported below. Our focus has been directed to neurodegeneration, particularly on Alzheimer's disease (AD). We are currently working on the identification of plasma biomarkers that might contribute to the stratification of patients affected by AD. Specifically, through a multidisciplinary approach integrating clinical investigations, plasma metabolomic analysis and cellular and molecular studies, our goal is to identify blood signatures that may help in the

stratification of AD patients, defining specific clinical phenotypes. This is crucial for the development of potential diagnostic and therapeutic personalized approaches.

PAPER 3
Serafini S., **Ferretti G**., Angiolillo A., Monterosso P., Di Costanzo A., Matrone
TNF-a levels are increased in patients with subjective cognitive impairment and are negatively correlated with amyloid beta 42.
Antioxidant, 2024 PMDI: 38397814

PAPER 4
Ferretti G*., Serafini S*., Angiolillo A., Monterosso P., Di Costanzo, Matrone
Advances in the identification of peripheral blood biomarkers in patients with Alzheimer's disease: moving closer to personalized therapies.
Biomedicine, 2023 PMID: 37392653

PAPER 5
Reveglia P., Paolillo C., Angiolillo A., **Ferretti G**., Angelico R., Sirabella R., Corso G., Matrone C., Di Costanzo A.
Targeting mass spectrometry approach to identify peripheral changes in the metabolic pathway from patients with Alzheimer's disease.
Int J Mol Sci, 2023 PMID: 34453619

PAPER 6
Reveglia P., Paolillo C., **Ferretti G**., De Carlo A., Angiolillo A., Nasso R., Caputo M., Matrone C., Di Costanzo A., Corso G.
Challenges in LC-MS-based metabolomics for Alzheimer's disease early detection: targeted approaches versus untargeted approaches
Metabolomics, 2021 PMID: 34453619

PAPER 7
Matrone C., Petrillo F., Nasso R., **Ferretti G.**
Fyn Tyrosine Kinase as Harmonizing Factor in Neuronal Functions and Dysfunctions
Int J Mol Sci, 2020 PMID: 32580508

PAPER 8
Brattico E., Bonetti L, **Ferretti G**., Vuust P., Matrone C.
Putting Cells in Motion: Advantages of Endogenous Boosting of BDNF Production
Cells, 2021 PMID: 33477654

ABBREVIATIONS

AD	Alzheimer’s disease
ADHD	Attention deficit hyperactivity disorder
ALS	Amyotrophic lateral sclerosis
ASD	Autism spectrum disorder
BBB	Blood-brain barrier
BSA	Bovine serum albumin
CD68	Cluster of differentiation 68
CD86	Cluster of differentiation 86
CNVs	Copy number variations
CRAM	CRMP-associated molecule
CRMP 2	Collapse response mediator protein 2
CSF	Cerebrospinal fluid
DAPI	4',6-diamidin-2-fenilindolo
DC	Dendritic cell
DOR	Decreased ovarian reserve
DPBS	Dulbecco's phosphate buffered saline
DRG	Dorsal root ganglia
DSM-5	Diagnostic and Statistical Manual for Mental Disorders
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
FAK	Focal adhesion kinase
FOV	Field of view
GAP	Gtpase-activating protein
GnRH	Gonadotropin-releasing hormone
GPI	Glycosylphosphatidylinositol
HH	Hypogonadotropic hypogonadism
Iba 1	Ionized calcium-binding adapter molecule 1
ID	Intellectual disability
IF	Immunofluorescence
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IPSc	Induced pluripotent stem cells
KO	Knock-out
LIMK	LIM kinase
MAM	Meprin/A5/μ-phosphatase
MAP	Microtubule-associated protein
MAPK	Mitogen-activated protein kinase
MeCP2	Methyl-cpg binding protein 2
MFI	Mean fluorescence intensity
MICAL	Molecules that Interact with casl
MS	Multiple sclerosis
NP	Neural progenitor
NRP	Neuropilin

PD	Parkinson’s disease
PDZ	Psd95/dig/zo-1
PLXN	Plexin
PSI	Plexin-semaphorin-integrin
RBD	Rho-gtpase binding protein
SEMA	Semaphorin
SNPs	Single nucleotide polymorphisms
SRM	Super resolution microscopy
STED	Stimulated Emission Depletion
TAOK2	Thousand-and-one kinase 2
TBS-T	Tris-buffered saline-tween
TK	Tyrosine kinase
TMEM 119	Targeting transmembrane protein 119
TNFα	Tumour necrosis factor α

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