Repulsive guidance molecule a (RGMa) induces degeneration of dopaminergic neurons in the mouse Substantia Nigra: implications for Parkinson's disease

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Abstract

Repulsive guidance molecule a (RGMa) is 2.5-fold upregulated in dopaminergic (DAergic) neurons of the SN in Parkinson’s disease (PD) (Bossers et al., 2009). siRNA-mediated knockdown of RGMa in SH-SY5Y neuroblastoma cells reduced cell viability, whereas overexpression of RGMa did not alter cell viability but reduced the neurite numbers formed by these cells (Chapter 4). Based on these results, we postulate that RGMa normally protects DAergic neurons from cell death, but that enhanced expression of this chemorepulsive protein may contribute to the retraction of nigrostriatal axons as observed in PD. To investigate the effects of RGMa on mesencephalic dopaminergic neurons in vivo, we overexpressed mouse RGMa in adult mouse SN DAergic neurons with the use of an adeno-associated viral vector harboring the RGMa coding sequence under the control of a synapsin promoter. Overexpression of RGMa in mesencephalic DAergic neurons resulted in: i) decreased tyrosine hydroxylase (TH) expression in the SN and in the striatum, ii) degeneration of SN neurons, iii) astro- and microgliosis, and iv) motor impairments that are characteristic of striatal dopamine deficiency. Interestingly, overexpression of RGMa did not affect the number of calbindin positive neurons in the SN pars compacta, a population of neurons that is also relatively preserved in PD. More work is required to unravel the mechanisms by which RGMa induces these neuropathological changes, but these preliminary data suggest that RGMa is a regulator of DAergic neuron survival and is a potential molecular target in the development of a regenerative treatment for PD.
Introduction

Repulsive guidance molecule, member a (RGMa), is a glycosylphosphatidylinositol-anchored glycoprotein that can be processed by extracellular proteases generating multiple membrane bound and soluble forms of this protein (Tassew et al., 2012). RGMa acts as repulsive axon guidance molecule in the developing amphibian, bird and mammalian central nervous system (CNS) (Monnier et al., 2002; Niederkofler et al., 2004; Samad et al., 2004; Matsunaga et al., 2006; reviewed in Mueller et al., 2006; and Yamashita et al., 2007). After traumatic brain and spinal cord injury RGMa is upregulated in infiltrating fibroblasts, reactive astrocytes and microglia, and it contributes to the repulsive environment of the CNS scar (Schwab et al., 2005a; Schwab et al., 2005b; Hata et al., 2006; reviewed in Mueller et al., 2006; and Yamashita et al., 2007). RGMa has been linked to multiple sclerosis by genetic association studies (Nohra et al., 2010) and was shown to be involved in the T cell-mediated pro-inflammatory response in a model for multiple sclerosis autoimmune encephalomyelitis (Muramatsu et al., 2011; Kubo et al., 2011). In contrast, during acute inflammation, RGMa was identified as an inhibitor of leukocyte migration (Mirakaj et al., 2011). Moreover, a novel chromosome microdeletion encompassing the RGMa gene has been linked in a single case study to the features of a neurogenetic developmental disorder called Angelman syndrome (Capelli et al., 2012).

RGMa, as well as netrin, both bind to the transmembrane receptor Neogenin (Itokazu et al., 2012). Neogenin is a so-called ‘dependence receptor’ (Mehlen and Bredesen, 2011). Dependence receptors mediate apoptosis in the absence of their ligand, while upon ligand binding they support proliferation, differentiation or cell survival (Matsunaga et al., 2006; Cole et al., 2007; Metzger et al., 2007; Lah and Key, 2012). Neogenin is cleaved by caspase-3 in the absence of RGMa and this initiates cellular apoptosis (Matsunaga et al., 2004; Matsunaga and Chedotal, 2004; Matsunaga et al., 2006). In an adult mouse retina injury model, intraocular injection of RGMa protein decreased caspase activity and reduced injury-induced retinal ganglion cell (RGC). Dependence receptor signaling appears therefore to play a key role in the protection of injured RGCs (Koeberle et al., 2010).

RGMa-Neogenin signaling also regulates growth cone collapse and axonal repulsion (reviewed in Wilson and Key, 2007; Yamashita et al., 2007). This occurs through the activation of RhoA/Rho-kinase and PKC, and is further mediated by myosin II phosphorylation leading to a reduction of F-actin in growth cones (Conrad et al., 2007; Kubo et al., 2008). Many neuronal cell types are sensitive to RGMa-Neogenin axon repulsive signaling, including cerebellar granule neurons (Hata et al., 2006; Kubo et al., 2008), embryonic cortical neurons (Yoshida et al., 2008), embryonic dorsal root ganglion cells (Conrad et al., 2007), enteric neuronal progenitors (Metzger et al., 2007) and embryonic retinal explants (Monnier et al., 2002; Tassew et al., 2012).
Two recent transcriptional profiling studies revealed that RGMa is upregulated in dopaminergic (DAergic) neurons of the substantia nigra (SN) of patients with Parkinson’s disease (PD) (Bossers et al., 2009; chapter 2; Neurocrine Biosciences, 2012). In a high content cellular screen (HCS, chapter 4), we showed that overexpression of RGMa in human DAergic neuroblastoma SH-SY5Y cells results in a small but significant reduction in the number of neurites per cell. This is consistent with RGMa’s function as a repulsive axon guidance molecule. In contrast, RGMa knockdown in the same SH-SY5Y cells decreased cellular viability and increased mitochondrial activity when cells were treated with the mitochondrial complex I inhibitor 1-methyl-4-phenylpyridinium (MPP(+)). Thus, whereas RGMa overexpression seems to reveal its axon repulsive function, RGMa knockdown appears to highlight the dependence receptor properties of Neogenin in the absence of ligand. Taken together, RGMa appears to play a protective role in cultured DAergic neuron-like cells, while it also exhibits a moderate repulsive activity on the processes formed by these cells.

Based on the upregulation of RGMa in the human SN and the functional data obtained in the HCS, we hypothesized that RGMa normally protects DAergic neurons in PD from cell death, but that axonal transport of enhanced levels of RGMa and secretion in the striatum would have a repulsive effect on synaptic terminals of the nigrostriatal pathway, thereby ultimately inducing neuronal loss in the SN. As a first step to test this hypothesis we overexpressed RGMa in adult mouse SN DAergic neurons with the use of an adeno-associated viral vector (AAV). Overexpression of RGMa induced neuronal degeneration in the SN and the loss of DAergic nigrostriatal axonal projections. Long-term overexpression of RGMa induced a movement disorder typical for a loss of DA in the striatum. These preliminary data suggest that RGMa is a regulator of the survival of DAergic neurons, and a potential molecular target in the development of a regenerative treatment for PD.

Methods

AAV constructs and viral vector production

The pAAV2Sna-SW and pTRUF20B-SEW plasmids, both generous gifts from Prof. Deniz Kirik (Lund University, Sweden), were used for cloning and production of AAV. Each plasmid contained two inverted terminal repeats of AAV2 flanking a human synapsin 1 (SYN) promoter driving expression of human α-synuclein (pAAV2Sna-SW plasmid) or GFP (pTRUF20B-SEW plasmid), followed by a woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) and a polyadenylation signal. For the construction of the empty vector (pAAV2-SYN) the pAAV2Sna-SW plasmid was cut with BamHI to remove the α-synuclein sequence and religated. For the construction of the vector containing mouse RGMa (pAAV2-SYN-RGMa) the pcDNA4/HisB-RGMaFL plasmid (a
A generous gift from Dr. J. Pasterkamp, Utrecht University, The Netherlands, was cut with Dra1 and Xho1 to isolate the full length mouse RGMa sequence and this fragment was ligated into the pAAV2Sna-SW backbone cut with EcoRV and Xho1.

Production of AAV7-Empty, AAV7-RGMa and AAV7-GFP viruses was performed using capsid and helper plasmids provided by J.M. Wilson (Gao et al., 2002). For each viral vector stock eight 15 cm petridishes containing 1x10⁷ human embryonic kidney 293T (HEK293T) cells were transfected with the use of polyethylenimine (PEI, MW25000; Polysciences Inc., Warrington, PA, USA). Cells were grown in Dulbecco’s modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (GIBCO-Invitrogen Corp, New York, NY, USA). pAAV2-SYN, pTRUF20B-SEW and pAAV2-SYN-RGMa plasmids were cotransfected with packaging plasmids in a 1:2:2 ratio (AAV-gene plasmid:helper plasmid pAdΔF6 : AAV7 capsid plasmid) with a total amount of 62.5 µg of DNA per plate. Two days after transfection cells were harvested in Dulbecco’s phosphate buffered saline (D-PBS) (Gibco) containing 10µg/ml DNaseI (Roche Diagnostics GmbH, Mannheim, Germany) and incubated for 1 hour at 37°C. Cells were lysed by three freeze-thaw cycles, spun down 30 min at 4000rpm and the crude lysate was collected. The virus was purified by iodixanol gradient ultra-centrifugation (Zolotukhin et al., 1999; Hermens et al., 1999) diluted in D-PBS/5% sucrose and concentrated using an Amicon 100kDA MWCO Ultra-15 device (Millipore, Billerica, MA, USA). Viral vector stocks were aliquoted and stored at -80°C until use. Titers were determined by quantitative PCR for viral genomic copies extracted from DNase-treated viral particles using WPRE directed primers (forward: CAGGTGTATTGCCACAAGACAAA and reverse: TGCA-CAGGTGAAGACCAAGCAA). The titer of the AAV7-Empty virus was 8.63x10¹² genomic copies/ml (GCs/ml), the titer of AAV7-GFP virus was 3.0x10¹² GCs/ml and the titer of the AAV7-RGMa virus was 9.71x10¹² GCs/ml.

**Experimental animals and surgical procedures**

A total of 38 male C57BL/6 mice weighing 20-25g (Harlan, Zeist, The Netherlands) were socially housed with food and water ad libitum, in 12 hour light and dark cycles. All the experimental procedures and postoperative care were carried out in accordance with the Institutional Animal Care and Use Committee of the Royal Netherlands Academy of Arts and Sciences.

The viral injections were carried out with the use of glass capillaries (0.78/1.0mm internal/external diameter; Harvard Apparatus, Holliston, MA, USA) with an 80µm tip connected via Portex polyethylene tubing to a Hamilton syringe fixed in a micro-infusion pump (PHD2000, Harvard Apparatus). The system was filled with water. The glass needles were mounted on a stereotactic device (David Kopf Instruments, Tujunga, CA, USA) and a total of 1.1µl of virus was loaded into the system. Two groups of animals were injected with different viral titers (Table 1).
Mice were intraperitoneally (IP) injected with an anesthetic mix consisting of Hypnorm (0.1 mg/kg Fentanyl citrate/3.3 mg/kg Fluanisone HCl, Janssen Pharmaceuticals) and Dormicum (8.3 mg/kg Midazolam, Roche) and placed into a stereotactic device where they were fixed and the skull was exposed. The skull was leveled using the heights of bregma, lambda and two lateral measurements 2.0 mm from bregma. The injection coordinates were -2.8 mm anterior posterior (AP) and -1.3 mm lateral (L) from bregma and -4.3 mm ventral dorsal (VD) from the dura. Subsequently, the needle was lowered into the brain 0.1 mm below the VD coordinate, retracted back up to the correct level and the infusion of 1µl at speed of 0.2µl/min was initiated. After the infusion, the needle was left in place for 3 min before retraction. Animals recovered from the anesthesia on a heating pad set to 37°C until fully recovered. Experimenters were blinded to the viral genotype.

**Statistical Analysis**

Unless mentioned otherwise, the Mann Whitney U test was used to estimate the differences between treatment groups. A P value < 0.05 was considered significant.

**Behavioural testing**

The behavior of 30 animals injected with the titer-matched viral batches (Table 1, experimental group 2) was assessed with the following tests: narrow beam test, grid test, cylinder test, swing test and tremor assessment. During the first 3 weeks post surgery, all tests were performed twice a week and subsequently once a week until week 12 post surgery. Baseline testing was performed 2 days before the surgery and the first test was performed 1 week after the surgery. The two investigators scoring the behavior tests were blinded for the treatment groups.

### Table 1. Experimental animal groups used in this study. Two experimental groups were used in this study. The viral vector, titer, number of animals and survival time of the animals is indicated for each experimental group.

<table>
<thead>
<tr>
<th>Experimental group 1</th>
<th>Experimental group 2</th>
</tr>
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<tr>
<td>Virus</td>
<td>AAV7-RGMa, AAV7-Empty</td>
</tr>
<tr>
<td>Titer</td>
<td>9.7x10^12</td>
</tr>
<tr>
<td>Number of animals per group</td>
<td>N=4</td>
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<tr>
<td>Survival time</td>
<td>3.5 weeks</td>
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## Experimental group 1

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<th>AAV7-RGMa, AAV7-Empty</th>
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</thead>
<tbody>
<tr>
<td>Titer</td>
<td>9.7x10^12</td>
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<tr>
<td>Number of animals per group</td>
<td>N=4</td>
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<td>3.5 weeks</td>
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## Experimental group 2

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<th>AAV7-RGMa, AAV7-Empty, AAV7-GFP, Saline</th>
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</thead>
<tbody>
<tr>
<td>Titer</td>
<td>3.0x10^12</td>
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<tr>
<td>Number of animals per group</td>
<td>N=8, for saline N=6</td>
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<td>Survival time</td>
<td>12 weeks</td>
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Grid test

The grid test was employed to study forepaw use, in particular the use of distal musculature and digit manipulation that are sensitive to dopaminergic input in the striatum (Tillerson and Miller, 2003). Mice were suspended upside down on a metal grid and allowed to move freely throughout the grid. For a successful trial, an animal had to hold on to the grid for a minimal 10 seconds and take at least 10 steps. The maximum trial length was 30 seconds. The number of steps with both forepaws was counted as well as the number of unsuccessful steps (overshoot, misgrab, grab with a wrist, loss of grip) with either forepaw by two blinded observers independently. Each animal performed three runs and the averaged ratio between the total forepaw faults/total forepaw steps over these three runs was calculated (Meredith and Kang, 2006).

Cylinder test

The cylinder test was performed to assess preference of front paw use during rearing behavior (Liu et al., 1999; Ulusoy et al., 2009). Animals were allowed to move freely in a glass cylinder for 5 minutes or until they performed 20 full rearing movements. For each rearing, it was recorded if the mouse used its left, right or both paws. The percentage of right paw use was calculated over the total number of the rearing score.

Narrow beam test

The hind limb placement was tested using the narrow beam test. Mice had to cross an 8 mm wide, 100 cm long and 15 cm elevated beam. The total number of correct hind limb steps and hind limb slips was counted and averaged over 3 complete runs. Two observers, blinded to the treatment, performed the scoring. Animals were pretrained for this test daily, a week before the surgery.

Tremor

A semi-subjective tremor assessment was performed during the narrow beam test when animals were stationary on the platforms. A positive tremor score required that the animal was shaking while stationary, had a shaky tail when stretched and showed an unstable and shaky front paw placement when exploring the environment.

Swing test

The swing test was performed as described by Iancu et al. (2005). The direction of body rotation was measured by suspending mice 5cm above the bottom of a cage while holding them at the base of their tail for 30 sec. During that time the direction of each swing above a 30° angle was scored.
**Tissue processing**

Animals were sacrificed at 3.5 weeks (Group 1, Table 1) or 12 weeks (Group 2, Table 1) after injection of the viral vector by an IP overdose with Pentobarbital (50mg/µl) and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde (PFA, Sigma-Aldrich Co., St. Louis, MO, USA) in sodium phosphate buffer (PBS, Sigma) pH 7.4. The brains were post-fixed overnight and 6 series of 30µm thick coronal sections were cut on a vibratome, containing both SN the striatum. The sections were stored free-floating at 4°C in 1% PFA in PBS pH 7.6.

**Fluorescent Immunohistochemistry**

All immunohistochemical (IHC) stainings were performed on free-floating sections. Prior to the staining, sections were blocked in blocking buffer (Tris buffered saline (TBS, Sigma) with 2.5% fetal calf serum (FCS, DAKO A/S, Glostrup, Denmark) and 0.2% Triton-X 100 (Sigma) for 1 hour (h) at room temperature (RT). Sections were incubated with anti-tyrosine hydroxylase (TH) antibody (either rabbit polyclonal (Pel-Freez Biologicals, AR, USA), or mouse monoclonal, clone LNC1 (Millipore MAB318, Temecula, CA, USA). The staining was combined with either anti-RGMa (D-16 (sc-46482), Santa Cruz, CA, USA) goat polyclonal antibody at 1:100, anti-glial fibrillary acidic protein (GFAP)-Cy3 conjugated mouse monoclonal antibody (G-A-5, Sigma, dilution 1:1500) or anti-Iba1 rabbit polyclonal antibody (activated microglia marker, Wako, Osaka, Japan, dilution 1:2000). Primary antibody incubations were performed in blocking buffer for 1h at RT followed by overnight incubation at 4°C. The following secondary antibodies were used for fluorescent staining: goat anti-rabbit/mouse/chicken Alexa 488 (1:800, Invitrogen, Carlsbad, CA, USA) for the detection of the TH antibody, donkey anti-goat Cy3 (1:800, Invitrogen) for the detection of RGMa and donkey anti rabbit DyLight (1:800, Invitrogen) for the detection of Iba1. These antibodies were incubated for 1h at RT in blocking buffer followed by 20 min incubation in PBS containing Hoechst 33258 (1:10000, BioRad, Hercules, CA, USA). Sections were then mounted on gelatin coated glass slides and embedded in Mowiol (0.1 M Tris pH 8.5, 25% glycerol, 10% w/v Mowiol 4-88 (Sigma)).

**Cresyl Violet and Calbindin Immunohistochemistry**

Sections were incubated with anti-calbindin D-28K mouse monoclonal antibody at dilution 1:500 (McAB 300, Swant, Switzerland). Primary antibody incubation was performed in blocking buffer for 1h at RT followed by overnight incubation at 4°C. For calbindin detection, sections were incubated with biotin labeled donkey anti-mouse antibody (1:400, Vector Laboratories, Burlingame, CA, USA) followed by an incubation with ABC Vectastain complex (1:800, Vector Laboratories) in 1xTBS for 1h. Finally, sections were incubated with DAB solution for 10min, washed in water, mounted and dried overnight at 37°C. Next day
sections were dehydrated and counterstained with 0.1% Cresyl Violet (Aldrich Chemical Company, Inc. Milwaukee, USA).

**Image processing and quantification**

Images were acquired with an AxioPlan 2 microscope (Zeiss, Sliedrecht, The Netherlands) with Planapochromat objectives, using Evolution QEi black and white or Evolution MP colour camera (MediaCybernetics) and ImagePro software. For each staining, all sections were imaged with the same exposure times.

**Estimation of neuronal density in the SN**

For each animal, one Cresyl violet and Calbindin stained series was used for cellular density measurement similarly as described by Bao et al. (2005), and Huitinga et al., (2000). Briefly, the entire SN structure in the non-injected side of the brain was identified at 2.5x magnification using the color camera on the Axioskop microscope. The anatomical borders of the non-injected SN were defined and outlined using the mouse brain atlas (Paxinos.G. and Franklin.K.B.J., 2001) and the outlined area was projected in a mirror fashion on the SN of the injection side, with slight adjustments to fit the anatomy of the contralateral SN. Additionally a smaller area in the post-thalamic nucleus just above the SN was outlined to serve as a comparison reference area. The outlined areas were subdivided into a rectangular grid using an Image Pro Plus macro, with each grid field representing one image at 40x magnification. For the SN, based on the standard deviation of the number of counted neurons per field, sampling of 35% of the total number of fields was sufficient to estimate the neuronal density. For the post thalamic nucleus, 100% of the fields were counted. Each neuron was identified based on the presence of a nucleus with a nucleolus and its morphological shape. Calbindin-positive and –negative neurons were counted separately. The total number of neurons in the two areas of interest from injected and non-injected side of brain was corrected for the size of the outlined area and thickness of each section to yield the average neuronal density in mm3. Based on these measurements, the decrease of neuronal density may also include neuronal atrophy, additionally to neuronal loss. Neuronal atrophy decreases the size of cells to be comparable to glial cells eliminating them from the counting criteria.
Results

Overexpression of RGMa decreases TH immunoreactivity in the SN pars compacta and striatum

High levels of RGMa expression were observed 3.5 weeks after unilateral injection of AAV-RGMa into the mouse SN in 3 out of 4 mice (Figure 1, Experimental group 1, Table 1). RGMa immunoreactivity was also observed in the striatum of 3 out of 4 AAV7-RGMa animals (Figure 2). We did not detect RGMa immunoreactivity in the SN of mice injected with the AAV7-Empty vector (Figure 1 and 2). In one animal injected with AAV7-RGMa (animal 2), RGMa expression was hardly detectable in the SN and in the striatum. In this animal, the injection of AAV7-RGMa apparently missed the SN pars compacta and some RGMa was present in the SN pars reticulata (Figure 1, animal 2).

High levels of RGMa expression in the injected SN were accompanied by a significant decrease of TH immunoreactivity as compared to the non-injected site (Figure 1). Additionally, we observed a significant decrease of TH immunoreactivity in the right striatum, the projection area of DAergic neurons transduced with AAV7-RGMa (Figure 2). Reduced TH immunoreactivity in both the SN and striatum was not observed in the AAV-Empty injected animals. Interestingly, in the non-injected SN and the striatum TH immunoreactivity was observed to be at higher level than in control AAV-Empty animals (Figure 1 and 2).

RGMa decreases neuronal density in the SN.

Although TH immunoreactivity was clearly down in the RGMa injected SN, we investigated whether this is due to downregulation of TH expression, or indeed due to loss of SN DAergic cells. Cresyl-violet staining of the mouse midbrain revealed a dramatic change of the cellular composition of the SN pars compacta in the animals overexpressing RGMa but not in the animals injected with AAV7-Empty (Figure 3). In animals injected with AAV-Empty, large neurons were present throughout the SN pars compacta. Overexpression of RGMa in the SN pars compacta resulted in a significant decrease in the number of large neuronal profiles in this area when compared to the contralateral SN or to the AAV-Empty injected animals (Figure 3C). There was no difference in neuronal density in the SN in AAV-Empty injected animals compared to the contralateral non-injected SN (p=0.35). Since RGMa expression was increased in a large area of the midbrain after transduction with AAV7-RGMa (Figure 1A), we next investigated if RGMa affected the neuronal density of the post thalamic nucleus (PTN). We observed a trend towards a decrease when comparing neuronal densities of the PTN in the AAV-RGMa injected midbrain to the contralateral PTN or to the AAV-Empty injected PTN, but this decrease was not statistically significant (p= 0.13 and p=0.09 respectively, Figure 3D).
Figure 1. Overexpression of RGMa in the mouse SN is associated with a decrease in TH expression. Immuno-histochemical staining for tyrosine hydroxylase (TH, green) and RGMa (red) in mouse SN injected with AAV-Empty (CTRL) or AAV-RGMa (RGMa) virus. The two panels for each animal represent the injected and non-injected SN pars compacta. Note the lack of TH staining in 3 out of 4 RGMa injected SN sections. RGMa animal 2 (A2) displayed TH immunoreactivity comparable to the CTRL animals. In this animal the injection of AAV7-RGMa appeared to have missed the SN. Arrow heads in this animal point to low levels of RGMa overexpression in some TH positive DAergic neurons of the SN pars compacta (upper arrow head) and in some neurons of the SN pars reticulata (lower arrow head). Scale bar represents 0.25mm.
Calbindin positive neurons in the SN of PD patients are less affected by the degeneration process as compared to the DAergic neurons (Yamada et al., 1990; German et al., 1992). It was therefore interesting to determine whether RGMa overexpression in the SN does affect calbindin positive neurons in the SN. RGMa overexpression did not significantly alter the number of calbindin positive neurons in the SN pars compacta when compared to AAV-Empty injection (p=0.62, Figure 3B and E), but there is a significant decrease in calbindin positive neuron density when comparing RGMa injected to non injected SN. Additionally, we observed an increase in diffuse calbindin immunoreactivity within the SN reticulata and around the SN pars compacta in RGMa-injected animals (Figure 2A), whereas in control animals calbindin immunostaining is confined to the SN reticulata with low levels detected in the lateral SN pars compacta (Figure 3A and B).

**RGMa overexpression activates astrocytes and microglial cells in the mouse SN**

In addition to the loss of neurons in the SN following overexpression of RGMa, we also observed an increase in the amount of small cells. To investigate whether RGMa overexpression had an effect on the glial response in the SN, we analyzed the expression of the astrocytic marker GFAP and of Iba1, a marker for reactive microglia. GFAP immunoreactivity showed only a slight increase in the SN pars compacta and reticulata injected with AAV-RGMa (Figure 4A and B). Additionally, there was a significant activation of microglia in the SN pars compacta transduced with AAV7-RGMa but not with AAV7-Empty virus (Figure 4C and D). Apart from RGMa associated gliosis, a slight increase of astrocytic reactivity was observed aligning the needle track in animals injected with AAV-Empty and AAV-RGMa. This was most probably caused by the mechanical damage inflicted by the penetration of the needle (Figure 4A and B, note in particular control animal 2).
Figure 3. Overexpression of RGMa decreased neuronal density in SN pars compacta. A. RGMa overexpression in the SN disrupted the anatomical structure of the SN. Arrowheads mark neurons aligned in the SN structure. In AAV-Empty injected control animals (CTRL A1 and 2) and in the misinjected AAV-RGMa animal 2 (RGMa A2), the SN neurons are compact and aligned on top of the SN pars reticulata (SN pars compacta-black arrowheads, SN reticulata-red arrow). In contrast, in RGMa overexpressing animals the cellular composition of the SN structure is disorganized (black arrowheads). Scale bar represents 0.25mm. B. Large magnification images of the lateral SN injected with AAV-Empty or AAV-RGMa virus. The large SN neurons are indicated by black arrowheads and calbindin positive neurons by white asterisk. Scale bar represents 0.1mm. C. RGMa overexpression decreases neuronal density in the SN. RGMa overexpression in SN pars compacta resulted in a decrease in the density of large neurons compared to the contralateral not injected SN and AAV-Empty injected SN. D. Neuronal density in the post thalamic nucleus (PTN) is not decreased after RGMa overexpression when comparing to AAV-Empty injected SN (p=0.09). E. RGMa overexpression did not affect the density of calbindin positive neurons in the mouse SN when comparing to the AAV-Empty injected SN (p=0.39), but there was a significant decrease in the density of calbindin positive neurons when comparing the RGMA injected to non-injected SN. Misinjected animal RGMa A2 was excluded from the statistical analysis. Due to low number of animals paired and non-paired student was used for all analyses (* p<0.05).
Figure 4. Glial response after RGMa overexpression in the mouse SN. A. GFAP immunoreactivity (red) was slightly increased in the RGMa injected SN. TH immunoreactivity is visualized in green. Increased levels of GFAP immunofluorescence can also be seen in the SN reticulata. Noteworthy, needle penetration of the tissue also induced a reactive astrogliosis, in particular in CTRL 2. B. High magnifications of panel A. C. Iba1 immunoreactivity (red), marking reactive microglia, indicated a vast reaction in the AAV-RGMa injected SN. Hoechst fluorescence (blue) additionally suggested a massive influx of cells in this area. D. High magnifications of panel C. Scale bars in A and C represent 0.25mm and in B and D 0.025mm.
**RGMa overexpression induces progressive motor impairments**

We next investigated whether overexpression of RGMa in the SN does also affect motor performance. Mice injected with AAV-RGMa in the SN (Experimental group 2, Table 2, with titer matched AAV injections leading to three times lower AAV-RGMa titer then in experimental group 1) developed behavioral motor deficits in the grid and cylinder tests (Figure 5). No deficits were observed in any of the control animals injected with either saline, AAV-Empty or AAV-GFP. The most striking behavioral deficit was revealed by the grid test, which is designed to measure accuracy of front paw placement and is significantly correlated with striatal dopamine levels (Tillerson and Miller, 2003). RGMa overexpressing animals performed progressively worse in this test ($p<0.001$), unlike the three control groups. The dysfunction started at 3.5 weeks and continued until almost the end of the experiment (Figure 5A). At week 12, RGMa animals showed a small degree of recovery, but performed still significantly worse than the AAV-GFP and saline injected animals.

In the cylinder test, overexpression of RGMa showed a significant progressive preference of right paw use during rearing behavior compared to either both paws or left paw only in time of the experiment (Figure 5B, $p<0.001$). At six time points (week 4, 6, 8, 10, 11 and 12), this preference was significantly different from the control groups. This suggests a dysfunction of the left front paw. Left front paw movements are controlled by the contra-lateral nigrostriatal system, which was injected with AAV-RGMa.

Although the error rate was slightly higher in the RGMa group, the narrow beam test did not reveal significant impairment of these mice in general motor function and hind limb placement skills (Figure 5C). The swing test also did not reveal a preference in turning behavior in any of the treatment groups (Figure 5D). A tremor assessment indicated more positive events within the AAV-RGMa injected animals. This behavior was highly variable within the AAV-RGMa injected group (Figure 5E), yet at week 6, RGMa overexpressing animals showed significantly more tremor compared to AAV-GFP and saline injected animals. Finally, RGMa overexpression had no impact on body weight (Figure 5F).

**Discussion**

We hypothesized that RGMa normally protect DAergic neurons from cell death, but that increased expression of RGMa, and axonal transport and secretion of this repulsive protein in the striatum would have a negative effect on synaptic terminals of the nigrostriatal pathway, subsequently inducing retrograde degeneration of mesencephalic DAergic neurons in the SN. AAV mediated overexpression of RGMa in adult mouse mesencephalic DAergic neurons resulted in i) reduced TH expression in the SN and in the striatum, ii) degeneration or atrophy of neurons in the SN, iii) astro- and microgliosis, and iv) a movement disorder.
Figure 5. Behavioral deficits after RGMA overexpressing in mouse SN. A. Grid test performance identified a progressive increase of front paw placement errors in the RGMA group (red) (Friedman test p<0.001) and not in the three control groups (AAV-Empty (blue), AAV-GFP (green) and saline (black). From week 3.5 until week 11 RGMA overexpressing mice showed significantly higher error rates compared to all three control groups (Mann Whitney U test, p<0.05). At week 12, RGMA mice performed worse compared to both GFP and saline animals (p= 0.009 and p=0.01 respectively), but not compared to the AAV-Empty animals (p=0.105). B. Preference of bilateral or unilateral front paw use was measured in a cylinder test. The ratio of right paw only rearing over the total rearing was determined. RGMA overexpressing mice progressively increased their right paw use during the time of experiment (Friedman test p=0.0001) compared to control groups. RGMA animals showed a significant use of raw paw compared to saline treated animals at week 4, 8 and 12 (black asterisk), to the AAV-Empty group at week 6 (blue asterisk), to saline and GFP at week 11 (green asterisk), and to all three control groups saline, Empty and GFP at week 10 (red asterisk). C. Hind limb placement was measured by narrow beam test. RGMA overexpressing mice showed more hind limb slip errors compared to AAV-GFP at week 7 and 11 (green asterisks). D. The swing test revealed no significant differences in the rotation preference of the mice between any of the treatment groups. E. Tremor was observed in RGMA overexpressing mice significantly more often at week 6 compared to AAV-GFP and saline injected animals. F. None of the treatment groups showed any significant differences in weight gain. For all tests,
characteristic of striatal dopamine deficiency indicating that RGMa has dramatic effects on the viability of SN DAergic neurons. More work is required to unravel the mechanisms by which RGMa induces these neuropathological and behavioral changes. Below we will discuss three likely possibilities.

Overexpression of RGMa induced degeneration of neurons in the SN - potential mechanisms

RGMa binding to its receptor Neogenin blocked the pro-apoptotic activity of Neogenin in chick embryos (Matsunaga et al., 2004; Matsunaga and Chedotal, 2004), and rescued adult RGCs from injury induced cell death (Koeberle et al., 2010). In a DAergic cell line, RGMa knockdown reduces cell viability, and this effect is amplified in cells with compromised mitochondrial function (chapter 4). Together, this supports a role of RGMa as a positive modulator of cell survival. In contrast, we show here that neuron-specific overexpression of RGMa in vivo in adult mouse SN neurons decreased TH expression in the nigrostriatal system and induced neuronal loss or atrophy. In order to understand the mechanism by which overexpression of RGMa leads to neuronal degeneration in the SN further studies are required. We envision three possible scenarios: i) axonal transport and secretion of increased amounts of RGMa from the SN to the striatum may induce a loss of nigrostriatal synaptic contacts, axonal retraction, and finally neuronal death and/or atrophy in the SN, consequently inducing a glial response, ii) overexpression of RGMa in DAergic neurons may result in enhanced secretion of RGMa in the SN where it directly activates astro- and microgliosis leading to the production of pro-inflammatory cytokines and subsequent neuronal death and/or atrophy, and finally iii) enhanced RGMa expression may induce both axonal repulsion in the striatum and a glial response, and those events may simultaneously lead to neuronal cell death and/or atrophy.

In support of the first scenario, following neuron-specific expression of RGMa in the SN we observed elevated RGMa protein levels in the ipsilateral striatum. RGMa immunoreactivity was distributed in a diffuse pattern in what appear to be thin fibers and extracellular matrix. This indicates that RGMa was most probably transported to the striatum via nigrostriatal projections. The presence of RGMa protein in the striatum may induce repulsive signaling in the DAergic nigrostriatal projections leading to synapse loss. Overexpression of RGMa in DAergic SH-SY5Y cells indeed decreased the number of neurites, supporting its function as a repulsive axon guidance regulator in DAergic cells (chapter 4). The induction of retrograde neuronal death and/or atrophy would
be in support of the dying back hypothesis (Dauer and Przedborski, 2003; Cheng et al., 2010). This hypothesis states that the primary neurodegenerative event in PD is the loss of dopaminergic nigrostriatal presynaptic terminals and the subsequent retraction of axons and degeneration of DAergic neurons in the SN. The glial response that we observe in the midbrain would thus be a secondary event induced by the neuronal cell death and/or atrophy.

In the second scenario, RGMa overexpressed in neurons is secreted locally in the SN and activates astrocytes and microglia in the SN leading to cytokine production and consequential neuronal death and/or atrophy. Indeed, RGMa immunoreactivity is observed in the extracellular space between the neuronal cell bodies of the SN after injection of AAV7- RGMa. Gliosis is known to occur in the PD SN (Langston et al., 1999; McGeer and McGeer, 2008), and may play a crucial role in the progression of neuronal degeneration by increased release of cytokines and chemokines (Hanisch, 2002; Hirsch et al., 2003; Barcia et al., 2003). Neogenin is expressed by microglia, macrophages and in the CD4+ T cells in the spinal cord (Hata et al., 2006; Muramatsu et al., 2011), and in the cells of blood vessels after lens injury and optic nerve crush (Schnichels et al., 2011). RGMa secreted by DAergic neurons in the mouse SN may therefore bind to neogenin expressed by these pro-inflammatory cells and this may result in cytokine and chemokine production, consequently inducing neuronal stress and neurotoxicity (Hanisch, 2002). RGMa may also bind to Neurogenin on blood vessels and T cells and attract more pro-inflammatory cells, a phenomenon that is observed in the EAE mouse model for MS (Muramatsu et al., 2011). RGMa-induced recruitment of pro-inflammatory cells could induce neuronal degeneration.

Finally, although we did observe a decrease in neurite numbers following RGMa overexpression in SH-SY5Y cells, cellular viability was not affected in these cells. RGMa signaling may thus induce both axonal repulsion in the striatum and glial activation in the SN, which together would lead to neuronal cell death (scenario 3). Alternatively, the lack of cell death in SH-SH5Y cells overexpressing RGMa may reflect a difference between the response of this cell line and DAergic neurons in vivo to RGMa.

The data presented here are insufficient to conclude which of these three mechanisms is implicated in the degeneration of SN DAergic neurons after RGMa overexpression. Firstly, it has to be determined which of the pathological events takes place first: loss of DAergic nigrostriatal presynaptic terminals and the subsequent retraction of axons, or gliosis and pro-inflammatory responses in the SN. Secondly, it should be established whether increased RGMa levels exert their effects on DAergic neurons in a Neogenin-dependent or –independent manner, e.g. by overexpression of RGMa in conditional Neogenin knockout mice.
**RGMa overexpression in mouse SN – similarities to PD etiology**

The effects of overexpression of RGMa in the mouse midbrain bear commonalities with the neuropathology of PD. As discussed above, overexpression of RGMa in the adult mouse SN decreases neuronal number and/or induces atrophy and activates astrocytes and microglia, both well known characteristics of PD. Additionally, RGMa overexpression induced movement deficits characteristic of DA loss in the striatum (Tillerson and Miller, 2003). These movement deficits developed in a progressive manner and lasted for at least 8 weeks, mimicking the progressive clinical symptoms in PD patients.

RGMa overexpression did not alter the density of calbindin positive neurons in the SN pars compacta, when compared to the AAV-Empty injection. Interestingly, in PD SN pars compacta the number of calbindin positive neurons does not decrease as dramatically as the calbindin negative SN neurons showing higher resistance to ongoing neurodegenerative process (Yamada et al., 1990; German et al., 1992). This effect is also observed in MPTP treated monkeys (Lavoie and Parent, 1991; German et al., 1992) and in a genetically induced PD mouse model, the aphakia mice (Luk et al., unpublished). We found a decrease of calbindin positive neurons in RGMa overexpressing SN when compared to the contralateral SN, but this effect is smaller than the decrease of total neuronal density, further arguing towards more resistance of calbindin neurons against the neurodegeneration. This decrease may also not be RGMA specific, since AAV-Empty injected SN also showed a trend towards a decreased calbindin positive neuron density, suggesting that the mechanical brain injection or neuronal AAV transduction may have an effect on the calbindin expression.

Besides the decrease in the number of neurons in SN, we found a slight trend towards a decrease of PTN neuron density after RGMa overexpression. Although, not significant, RGMa signaling may be activated in PTN neurons, leading to a decreased neuronal survival or neuronal atrophy. As we have shown only an upregulation of RGMa in the PD SN (Bossers et al., 2009), and not studied the PTN in PD, we cannot judge the possible role of this effects in translation to PD pathology. Nevertheless, it is noteworthy that RGMa may have a more broad effect on midbrain neurons, besides the DAergic neurons of the SN.

To further investigate the commonalities between overexpression of RGMa in the mouse midbrain and neuropathology of PD, we aim to study other features that are prominent in PD, such as Lewy body formation, and mitochondrial dysfunction. If these aspects of PD pathology are also present in RGMa overexpressing mice, this will further contribute to the relevance and importance of this target gene in the development and/or progression of PD.