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Review

Chromatolysis: Do Injured Axons Regenerate Poorly when Ribonucleases Fragment or Degranulate Rough Endoplasmic Reticulum, Disaggregate Polyribosomes, Degrade Monoribosomes and Lyse RNA?

Running title: Which ribonucleases limit axon regeneration?

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Abstract: After axonal injury, chromatolysis (fragmentation of Nissl substance) occurs in both intrinsic neurons (whose processes are within the CNS) and extrinsic neurons (whose axons extend outside the CNS). Electron microscopy shows that chromatolysis involves fission of the rough endoplasmic reticulum. In intrinsic neurons (which do not regenerate axons) or in extrinsic neurons denied axon regeneration, chromatolysis is often accompanied by degranulation (loss of ribosomes from rough endoplasmic reticulum), disaggregation of polyribosomes and degradation of monoribosomes into dust-like particles. Ribosomes and rough endoplasmic reticulum may also be degraded in autophagic vacuoles by Ribophagy and Reticulophagy, respectively. In other words, chromatolysis is disruption of parts of the protein synthesis infrastructure. Whereas some neurons may show transient or no chromatolysis, severely injured neurons can remain chromatolytic and never again synthesise normal levels of protein; some may atrophy or die. What molecule(s) cause fragmentation or degranulation of rough endoplasmic reticulum, disaggregation of polyribosomes and degradation of monoribosomes? Ribonucleases can modify (and perhaps fragment) rough endoplasmic reticulum; various endoribonucleases can degrade mRNA causing polyribosomes to unchain and disperse; they can disassemble monoribosomes; Ribonuclease 5 can control rRNA synthesis and degrade tRNA; Ribonuclease T2 can degrade ribosomes, rough endoplasmic reticulum and RNA within autophagic vacuoles; and Ribonuclease IRE1α acts as a stress sensor within the endoplasmic reticulum. Regeneration might be improved after axonal injury by protecting the protein synthesis machinery from catabolism; targeting ribonucleases could be a profitable strategy.

Keywords: chromatolysis, ribonuclease, angiogenin, endoplasmic reticulum, ribosome

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Injury to mammalian axons can cause a transient or persistent impairment in protein synthesis.

Why does axonal injury result variably in axon regeneration or collateral sprouting, atrophy or cell death (Thuret *et al.*, 2006)? A long-standing observation is that after axonal injury, PNS and CNS neurons can undergo “chromatolysis” (Torvik and Heding, 1969; Lieberman, 1971; Egan *et al.*, 1977a; Egan *et al.*, 1977b; Barron, 1983; Barron, 2004; Severinsen and Jakobsen, 2009; Johnson and Sears, 2013). This catastrophic event involves dramatic whole-cell morphological changes that are easily visible under the light microscope (*e.g.*, after cresyl violet or toluidine blue staining for Nissl substance). Its hallmarks are changes in the aggregation, organisation and location of “Nissl bodies" as seen under the light microscope (Figure 1) (Lieberman, 1971). Electron microscopy reveals that Nissl bodies are parallel arrays of cisterns of rough endoplasmic reticulum studded with ribosomes; rosettes of free polyribosomes and monoribosomes are found between the cisterns (Figure 2) (Matthews and Raisman, 1972; Johnson and Sears, 2013). Each ribosome is a complex of ribosomal RNAs (rRNAs) and proteins that use transfer RNAs (tRNAs) and amino acids to synthesise proteins from mRNAs. In other words, Nissl bodies are a major part of the protein synthesis machinery of a neuron.

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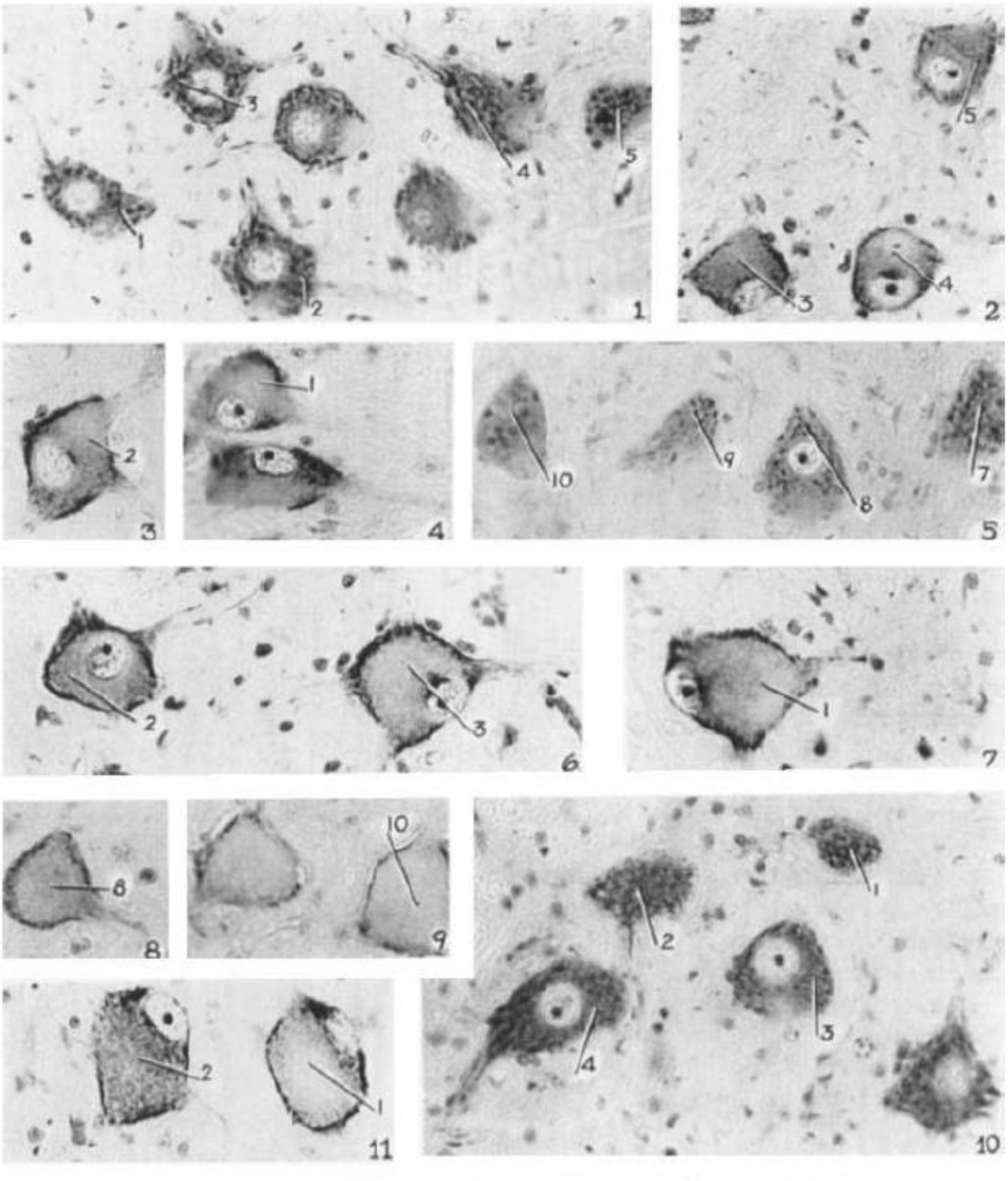

Figure 1: Chromatolysis in neurons involves gross structural abnormality of Nissl substance. Images of toluidine blue-stained sections of adult monkey cervical spinal cord showing motor neurons after section of a dorsal and ventral root at lumbar or sacral levels. Subpanels 1, 5 and 10 show uninjured neurons. Subpanels 2-5 show sections 3 days after injury and subpanels 6-9 show sections 6 days after injury. Subpanel 11 is 10 days after injury. [mages taken from (Gersh and Bodian, 1943); magnification is X 250].

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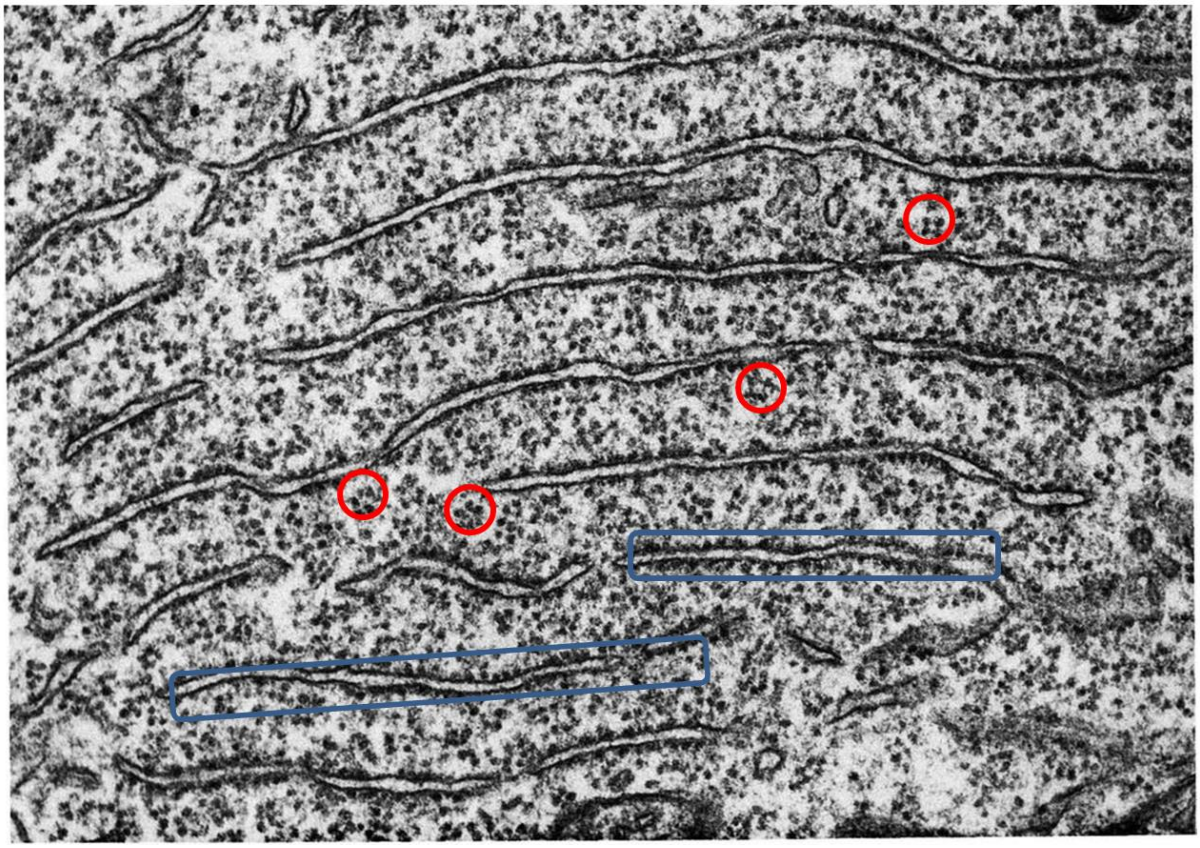

Figure 2: Electron microscopy shows that Nissl bodies in a motor neuron are stacks of rough endoplasmic reticulum whose cisterns are studded externally with ribosomes (blue regions) and interspersed with rosettes of polyribosomes (red circles). [Image taken from Palay in (Fawcett, 1981) (p.319) in which magnification is not stated but it was noted that fenestrated cisternae are separated by intervals of 0.2 to 0.5 μm].

EM shows that chromatolysis is the fragmentation of stacks of rough endoplasmic reticulum leaving clear areas of cytoplasm lacking Nissl bodies; in some cases (see below) this can be accompanied by the disaggregation and/or disassembly of polyribosomes to leave a fine ("dust-like") powder (Cragg, 1970; Matthews and Raisman, 1972; Torvik, 1976; Barron and Dentinger, 1979; Dentinger *et al.*, 1979; Johnson and Sears, 2013). Ribosomes can be depleted from rough endoplasmic reticulum (Lieberman, 1971; Barron, 1989). This can be accompanied by the degradation of monoribosomes (Lieberman, 1971; Engh and Schofield, 1972; Torvik, 1976). Ribosomes and fragments of endoplasmic reticulum can also be found in autophagic vacuoles (Matthews and Raisman, 1972; Torvik, 1976). The cell body response can also involve dispersion to the soma's periphery of any remaining ribonucleoprotein complexes (Cragg, 1970; Barron and Dentinger, 1979; Dentinger *et al.*, 1979; Johnson and Sears, 2013) (Figure 3) and movement of the nucleus to an eccentric position. In other words, chromatolysis is the visible disarray of key parts of the protein synthesis machinery (Lieberman, 1971).

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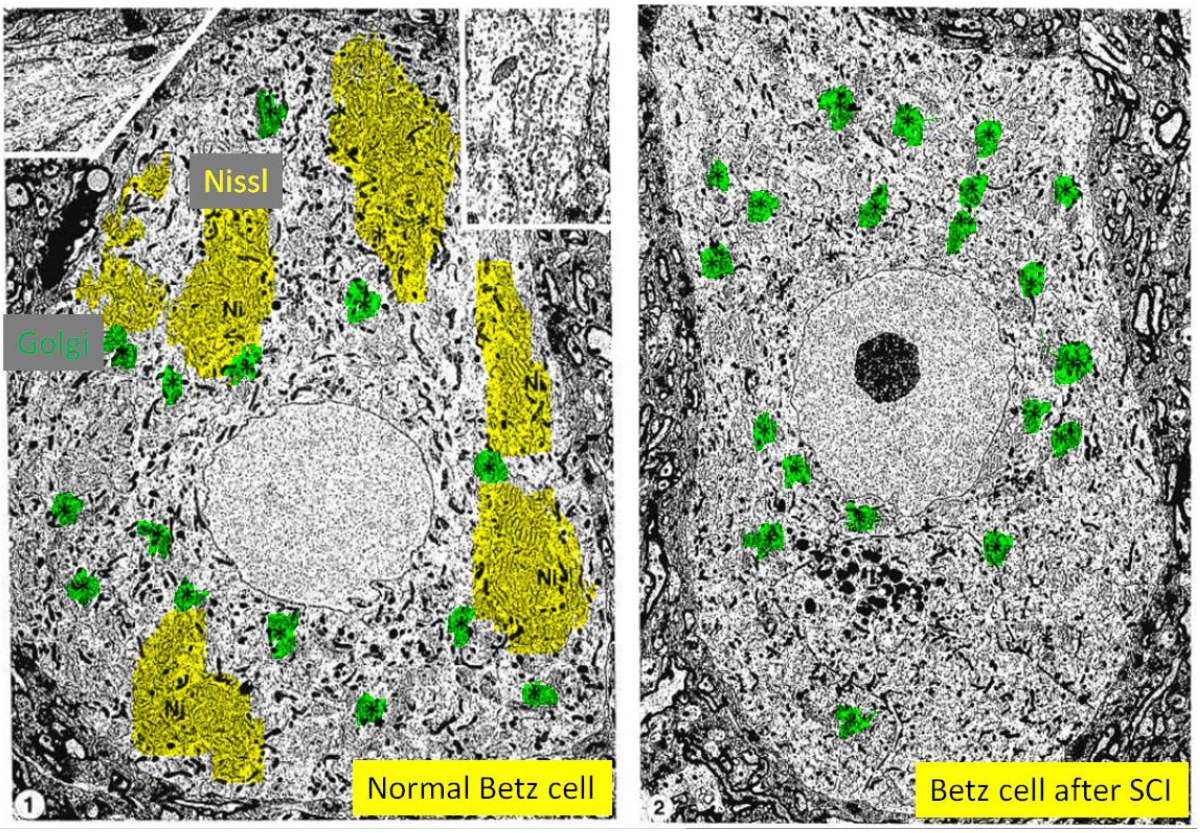

Figure 3: Chromatolysis in CNS neurons involves destruction of the protein synthesis machinery. Electron microscope image showing Betz neurons from pericruciate cortex of either (panel 1) a normal adult cat or (panel 2) an adult cat 10 days after spinal cord injury (C2 lateral funiculotomy). Nissl substance is highlighted in yellow (Ni) and aggregates of Golgi are highlighted in green (*). Normal Nissl is no longer visible in the cortical neuron after spinal cord injury. [Images from (Barron and Dentinger, 1979); magnification of panel 1 is X 5,300 (inset is X 21,700) and magnification of panel 2 is X 3,400].

Although early investigators regarded chromatolysis as a regressive event, others noted the reversible nature of chromatolysis during successful axon regeneration (Gersh and Bodian, 1943). Some reviews of chromatolysis in the early 1970s concluded that chromatolysis is essential for (and enables) axon regeneration (Cragg, 1970; Torvik, 1976), whereas others proposed that chromatolysis is a catabolic process which can overlap in time with other anabolic processes (Engh and Schofield, 1972; Matthews and Raisman, 1972) such as rRNA synthesis for production of new ribosomes. However, such papers were largely based on data from extrinsic neurons capable of axon regeneration including spinal motor neurons, DRG neurons injured peripherally and sympathetic cervical ganglia (SCG) neurons injured post-ganglionically (Cragg, 1970; Lieberman, 1971; Watson, 1974). In these neurons, after crush injury (which allows regeneration), levels of total RNA or of newly synthesised total RNA generally increase at most times after injury showing that the anabolic processes generally exceeds the catabolic processes of chromatolysis in extrinsic neurons (Watson, 1968; Lieberman, 1971) but not in intrinsic neurons (Barron, 1989). However, the proportion of total RNA that is mRNA is small (typically <5%) compared to rRNA and therefore much of the rise may be dedicated to production of new ribosomes. Because rRNA is not translated,

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measuring uptake of radiolabelled protein precursors can inform one about changes in protein synthesis after injury (Lieberman, 1971; Barron, 1989). Various experiments show using extrinsic neurons that transection (which prevents regeneration) rather than crush (which allows regeneration) can cause persistent chromatolysis (Johnson *et al.*, 1985; Johnson *et al.*, 1993), subnormal neuronal RNA levels, and reduced protein synthesis [(Watson, 1968; Kung, 1971) and see references in (Lieberman, 1974; Torvik, 1976; Barron, 1983)]. For example, large DRG neurons show a ~50% decrease in protein synthesis between 1 and 35 days after sciatic crush and persistently decreased protein synthesis from 1 to 95 days after sciatic transection (Engh *et al.*, 1971). However, there are counterexamples showing generally increased protein synthesis after crush of extrinsic neurons [(Lieberman, 1971) but see (Kung, 1971; Engh and Schofield, 1972; Barron, 1983)].

Data published in the later 1970s and 1980s proved that chromatolysis in (non-regenerative) intrinsic neurons involves a reduction in protein synthesis with disassembly and dispersal of the RER (Torvik and Heding, 1969; Barron *et al.*, 1976; Barron *et al.*, 1977; Barron *et al.*, 1982; Barron *et al.*, 1989). Thus, in general, axotomised extrinsic neurons show increased rates of uptake of radiolabelled amino acids whereas axotomised intrinsic neurons show reduced rates of uptake (Barron, 1989). Specifically, intrinsic neurons undergoing chromatolysis (*e.g.*, after spinal cord injury) show reduced levels of RNA in the nucleolus and cytoplasm and reduced protein synthesis per cell within and beyond 24h (Barron *et al.*, 1976; Barron *et al.*, 1977; Barron *et al.*, 1982; Barron, 1983; Barron, 1989; Barron *et al.*, 1989). This is in marked contrast to extrinsic neurons which undergo brief chromatolysis that rapidly becomes accompanied by an anabolic phase that re-assembles the protein synthesis machinery resulting in axon regeneration (Cragg, 1970; Matthews and Raisman, 1972). Chromatolytic intrinsic neurons of mammals show disaggregation of free, clustered polyribosomes into single units and degranulation of cisternal membranes whereas chromatolytic extrinsic neurons retain clusters of free polyribosomes unless cell death supervenes (Barron, 1983; Barron, 1989). Stressed neurons also form stress granules which may be 100 to 200 nm in size, lack a surrounding membrane and are composed of proteins and RNAs. Stress granules can be a site for degradation of mRNAs or storage of mRNAs until the period of stress has passed (Wolozin, 2012). Not all neurons undergo dramatic chromatolysis after injury (Claman and Bernstein, 1981); this depends on the age of the subject, the type of injury (e.g., crush vs transection), whether the injury is distal from or proximal to the cell body and whether there are spared collaterals proximal to the injury site (Lieberman, 1974; Barron, 2004).

In the 2000s and 2010s, transcriptomic or proteomic experiments have not usually reported a global suppression of protein synthesis in homogenates of neurons and glia after PNS crush although the expression level of many transcripts and proteins do go down. It may be relevant that many of these experiments have been normalised in a way that might mask overall changes in protein synthesis (*e.g.*, RNA sequencing experiments are often normalised to the FPKM; number of fragments per kilobase million). However, key gene changes are generally corroborated by qRTPCR data (normalised to an invariant mRNA) or cell-type specific *in situ* hybridisation data. Nonetheless, in the 2020s, it will be useful to use single-cell-type sequencing to determine which, if any, injuries induce global reductions in protein synthesis during phases of chromatolysis on a per-neuron basis. Again, it is important to emphasise that not all injuries cause chromatolysis, and that the catabolic consequences of chromatolysis

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may not cause an overall (net) loss in protein synthesis capacity if the anabolic response is quick and strong: considering all the available data, protein synthesis appears to increase in neurons that sprout or regenerate.

In conclusion, given the ultrastructural hallmarks of chromatolysis (e.g., fission or dispersal of the RER and disaggregation of polyribosomes), the most plausible explanation is that the functional consequence of chromatolysis is disruption of protein synthesis which can be transient or permanent depending on a variety of factors including the type and the location of injury.

Do ribonucleases cause fragmentation, dispersal and degranulation of rough endoplasmic reticulum, dissociation of polyribosomes and degradation of monoribosomes and RNA after injury?

Perhaps amazingly, given that chromatolysis was first reported in the late 1800s [by Nissl in 1892 and Marinesco in 1898; (Severinsen and Jakobsen, 2009)], it is not yet known what molecule(s) fragment and degranulate rough endoplasmic reticulum, disaggregate polyribosomes and degrade monoribosomes. In 1943 Gersh and Bodian proved (using light microscopy) that Nissl substance in spinal motor neurons contains RNA by showing that treatment of fixed spinal cord sections with ribonuclease entirely abolished subsequent Nissl staining [Figures 14-16 in (Gersh and Bodian, 1943)]. They went further and suggested that chromatolysis might occur due to the activity of ribonucleases *in vivo*. In the 1960s, it was shown that chromatolysis in injured facial nerve neurons requires new protein synthesis (Torvik and Heding, 1969) and the authors wondered whether an enzyme might be responsible for dispersion of the Nissl substance (Torvik and Heding, 1969).

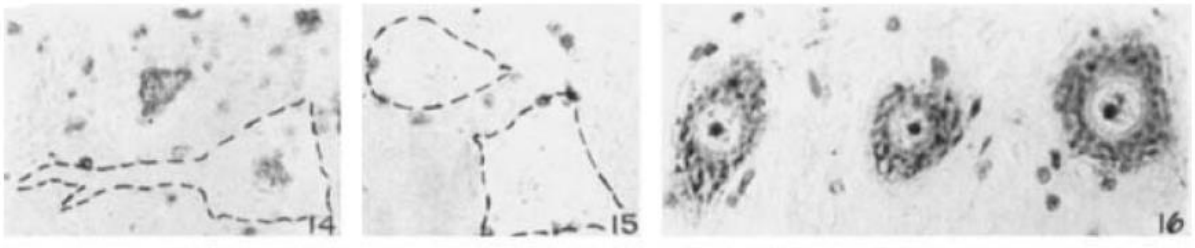

Figure 4: Nissl substance (*i.e.*, rough ER) can be destroyed with ribonuclease. L7 spinal cord neurons stained for Nissl using toluidine blue either without (subpanels 14, 15) or with (subpanel 16) treatment of fixed tissue sections with ribonuclease. The cell bodies are outlined with broken lines. Nissl bodies are visible as dark patches in the cytoplasm in subpanel 16 but are not visible in subpanels 14 or 15. Nucleolar basophilic staining is also nearly abolished. Staining of chromatin of glia is not affected. [Image taken from (Gersh and Bodian, 1943); magnification is X 250].

In 1970, Cragg asked "What is the signal for chromatolysis?" and he considered various hypotheses including signals conveyed by retrograde transport from the site of injury. However, he concluded "The hypothesis that the neurone produces a substance that represses neuronal RNA production, and loses some of this repressor when the axon is injured or when it sprouts, comes nearest to explaining the experimental findings as they are known at present". With the benefit of hindsight, this conclusion might explain the anabolic response

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seen in chromatolytic extrinsic neurons that regenerate, but it cannot explain the catabolic response seen in chromatolytic intrinsic neurons that were described after 1970. Since Cragg's review, many groups have described retrograde signals from injury sites that can induce neuronal cell body responses (Hanz *et al.*, 2003; Hanz and Fainzilber, 2006; Ying *et al.*, 2014; Hu, 2016). Here, I consider the possibility that the catabolic response is executed in part by various ribonucleases; the anabolic mechanism whereby the protein synthesis machinery is built or rebuilt is beyond the scope of this review (Olson and Dundr, 2015).

From the 1970s, there are beautiful ultrastructural images of sympathetic ganglia undergoing chromatolysis after postganglionic injury (Matthews and Raisman, 1972). Biochemical experiments in the 1980s then revealed that uninjured sympathetic ganglia contain inactive ribonucleases [see references in (Bates *et al.*, 1985b)] and that the total activity levels of alkaline ribonucleases increase in sympathetic ganglia after postganglionic nerve injury: this is the result of increased synthesis of ribonucleases as well as activation of existing ribonucleases. This activity becomes progressively restrained by one or more endogenous Ribonuclease Inhibitors (Bates *et al.*, 1985a, b; Bates *et al.*, 1987). This is consistent with the idea that chromatolysis in sympathetic ganglia is due to ribonuclease activity and that any anabolic response occurs after progressive inhibition of cytoplasmic ribonucleases. [An increase in nuclear ribonuclease activity may be required for processing of newly synthesised RNA in the anabolic phase (Bates *et al.*, 1987; Pizzo *et al.*, 2013)]. To my knowledge, these ribonucleases and ribonuclease inhibitors have not been studied to any great extent in DRG neurons or CNS neurons.

In the 1980s it was suggested that some "suicide enzyme", perhaps a "powerful ribonuclease", is responsible for disassembly of polyribosomes (Rubel *et al.*, 1991) into dust-like particles in a developing chick CNS nucleus (*nucleus magnocellularis*) deprived of all afferent input (Rubel *et al.*, 1991). [Because RER was not seen to be degraded in this study, other mechanisms may be responsible for this phenomenon; see below]. Polyribosomes are also turned to dust in severely injured chromatolytic sympathetic ganglia (Matthews and Raisman, 1972) and in dendrites of uninjured dentate gyrus neurons after treatment of fixed hippocampal tissue blocks with ribonuclease of "type II" (Sigma) (Steward, 1983). Indeed, because polyribosomes are linked together by mRNA, ribonuclease treatment can degrade mRNA and dissociate them [(Warner *et al.*, 1963; Gerashchenko and Gladyshev, 2017) and see p. 305 in (Fawcett, 1981)].

If ribonucleases can disassociate polyribosomes into monoribosomes, what causes degradation of monoribosomes? Every ribosome is made of two subunits each composed of a complex of rRNAs with proteins; indeed, rRNA comprises the predominant material by weight (comprising ~60% of the ribosome mass). It is not surprising, therefore, that exogenous treatment using various ribonucleases can degrade monoribosomes into "dust-like" fragments *in vitro* (Steward, 1983; Rubel *et al.*, 1991) [see also (Blasi *et al.*, 2000; Gerashchenko and Gladyshev, 2017)]. Interestingly, endogenous ribonucleases accompany ribosomes (Bransgrove and Cosquer, 1978; Bates *et al.*, 1985a; Bates *et al.*, 1987; Schoenberg and Maquat, 2012) including in the adult mammalian brain (Datta and Ghosh, 1962) presumably constrained by an endogenous inhibitor (Allam *et al.*, 2017) (see below). As will be seen next, particular ribonucleases are plausibly responsible for fragmentation of rough endoplasmic reticulum, dissociation of polyribosomes, degradation of monoribosomes and decay of RNA.

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Which ribonuclease(s) might cause fragmentation of rough endoplasmic reticulum, disassociation of polyribosomes, degradation of RNA and monoribosomes after axotomy?

There are many Ribonucleases including some that are optimally active under acidic conditions (pH 4 to 5) such as Ribonuclease T2 (Haud *et al.*, 2011) (see below), whilst others such as those in the RNase A family are optimally active at alkaline (pH 7-8) or weakly acidic conditions (pH 6.5-7) (Luhtala and Parker, 2010). Both alkaline (Bates *et al.*, 1985a, b; Bates *et al.*, 1987) and acidic (Haud *et al.*, 2011) ribonucleases may play a role in chromatolysis. There are a variety of stress-induced ribonucleases such as those in the secreted, vertebrate ribonuclease family (Ivanov and Anderson, 2011; Nicholson, 2011) whose canonical member is bovine pancreatic RNase A (often known as Ribonuclease 1 or pancreatic RNase); in humans there are eight canonical Ribonucleases in this family. RNases have different specificities and may play different roles after neuronal injury. For example, Ribonuclease 5 cleaves tRNA (but not mRNA or rRNA) whereas others including Ribonucleases 1 and 2 cleave mRNA, rRNA and tRNA (Saxena *et al.*, 1992).

Given the evidence that ribonucleases can accompany ribosomes (Datta and Ghosh, 1962; Bransgrove and Cosquer, 1978; Bates *et al.*, 1985a; Bates *et al.*, 1987; Schoenberg and Maquat, 2012), it is possible that they become activated after injury (Allam *et al.*, 2017). Ribonucleases may also become activated after they change their subcellular distribution after axotomy. Data obtained using non-neural cells show that under conditions of stress, Ribonuclease 5 moves to the cytoplasm including stress granules where it becomes activated and hemisects tRNAs into tiRNAs: this impairs translation of proteins, save many essential for cell survival (Pizzo *et al.*, 2013). Finally, there is one report that chromatolysis can be prevented by protein synthesis inhibitors (Torvik and Heding, 1969) indicating that chromatolysis may be due to a newly-synthesised catabolic enzyme; accordingly, perhaps ribonucleases synthesised in response to neuronal injury contribute to chromatolysis (Bates *et al.*, 1985a; Mami *et al.*, 2016).

Many ribonucleases are also secreted and are found in cerebrospinal fluid (CSF) (Yasuda *et al.*, 1993) including Ribonuclease 2, Ribonuclease 3 (Eosinophil Cationic Protein; ECP) and Ribonuclease 5 (also known as Angiogenin) (Ng *et al.*, 2011) and increased levels are found in CSF and blood after spinal cord injury in humans including Ribonuclease 5 (Rabin *et al.*, 1977; Ng *et al.*, 2011). Several (but not all) Ribonucleases cause neuronal injury when given intrathecally including Ribonucleases 2 and 3 (Newton *et al.*, 1994). Indeed, Ribonuclease 2 is also known as Eosinophilic Derived Neurotoxin (EDN); it causes rapid neuronal cell death when it is injected intrathecally (Sorrentino *et al.*, 1992; Newton *et al.*, 1994). Injection of various Ribonucleases, including 1 and 5, into cells results in the degradation of the cells' RNA and causes cell death (Saxena *et al.*, 1991; Saxena *et al.*, 1992). Thus, it is possible that chromatolysis may be due to uptake of a ribonuclease from the extracellular environment after injury; however, this would need to be reconciled with the fact that chromatolysis tends to start centrally, sometimes (but not always) with sparing of peripheral rims of Nissl (Barron, 2004).

1. Which ribonuclease(s) might cause fragmentation of rough endoplasmic reticulum?

It is not known what causes fragmentation or disarray of rough endoplasmic reticulum in chromatolytic neurons. However, extensive and rapid fission of endoplasmic reticulum in

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CNS dendrites can be triggered by increases in intracellular calcium *in vitro* and in adult cortical neurons during global ischemia *in vivo* (Kucharz *et al.*, 2009; Kucharz *et al.*, 2011, 2013; Zhao and Blackstone, 2014). Interestingly, this process is reversible: fusion of fragments occurs if the fissile stimulus (*e.g.*, K^+) is washed out or if an NMDA receptor antagonist is applied (Kucharz *et al.*, 2013). It is not yet known whether smooth endoplasmic reticulum in the axon or rough endoplasmic reticulum in the cell body also undergoes fission under these circumstances (Kucharz *et al.*, 2013).

The mechanism(s) by which endoplasmic reticulum is fragmented is not known but there is evidence from other cell types that calcium-dependent ribonucleases in the EndoU/PP11 family dynamically regulate endoplasmic reticulum (Zhao and Blackstone, 2014). In *Xenopus* oocytes XendoU is bound to the endoplasmic reticulum where it can degrade RNA and remove ribosomes and ribonucleoproteins. This causes expansion of rough endoplasmic reticulum at the expense of smooth endoplasmic reticulum: depletion of XendoU caused an expansion of rough endoplasmic reticulum sheets at the expense of smooth endoplasmic reticulum tubules which could be rescued by XendoU in a ribonuclease-dependent manner (Schwarz and Blower, 2014). However, it is not clear whether EndoU/PP11 family members cause fragmentation *per se* rather than switching of ER type from rough to smooth by degranulation. Ribonuclease 1 can also cause dose-dependent changes in endoplasmic reticulum in non-neural cells (Schwarz and Blower, 2014). Interestingly, there is one report of depletion of rough endoplasmic reticulum with expansion of smooth endoplasmic reticulum in injured adult cat red nucleus neurons after spinal cord injury (Barron *et al.*, 1975) but increases in smooth endoplasmic reticulum after injury to intrinsic or extrinsic neurons has not been reported more widely (Barron, 1983). It will be important to determine whether calcium-dependent ribonucleases cause degranulation and/or fragmentation or depletion of rough endoplasmic reticulum in neuronal cell bodies.

2. Which ribonuclease(s) might cause disassociation of free polyribosomes or degradation of monoribosomes?

Treatment of purified ribosomes (from mouse liver) with Ribonuclease 1 causes disassembly of polyribosomes (into monoribosomes) and degradation of monoribosomes *in vitro* whereas treatment with other ribonucleases (e.g., T1) only causes disassembly of polyribosomes into monoribosomes and does not cause degradation of monoribosomes into fragments (Gerashchenko and Gladyshev, 2017) [*n.b.*, T1 is a fungal ribonuclease so some other ribonuclease would have to be responsible in mammals (Blasi *et al.*, 2000)].

During cellular stress, when eukaryotic initiation factor 2α (eIF2α) is phosphorylated, it inhibits formation of a complex containing EIF2, GTP and $tRNA_i^{Met}$ which leads to formation of stress granules in which mRNAs may be stored or degraded (Wolozin, 2012). Stress also causes “No-go decay” and “Nonsense mediated decay” of mRNAs by ribonucleases that release ribosomes from endoplasmic reticulum (Schoenberg and Maquat, 2012). Stress granules may contain ribonucleases that can cleave mRNAs, including Polysome-Bound Endonuclease (PMR1) and GTPase-activating protein binding protein (G3BP-1), or that can cleave tRNAs including Ribonuclease 5. Stress granules can also be induced in an eIF2α-independent manner by Ribonuclease 5 (Emara *et al.*, 2010). However, these ribonucleases have not much been studied in axotomised neurons.

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Why don't endogenous, potent Ribonucleases constitutively cause chromatolysis in uninjured neurons?

The activities of ribonucleases are constrained in various ways including activation of ribonucleases by phosphorylation (e.g., PMR1) or oligomerization (e.g., IRE1α) (Schoenberg and Maquat, 2012). XendoU is activated by calcium entry (which happens after axotomy or during ER stress). Other ribonucleases are inactivated in the reducing environment of the normal cell and become active under conditions of oxidative stress. The secreted vertebrate family of ribonucleases is normally tightly controlled by the mammalian Ribonuclease Inhibitor 1 (RNH1) (Dickson *et al.*, 2005). RNH1 is found primarily in the cytoplasm, where it binds to these RNAses with remarkably high affinities (Dickson *et al.*, 2005) and inhibits their activities, although it can also be found in the nucleus, in mitochondria and in stress granules (Furia *et al.*, 2011). Cytoplasmic inhibition of ribonucleases is disrupted in cellular stress situations, *e.g.*, by oxidative stress (Dickson *et al.*, 2005), during which RNH1 is translocated to the nucleus and Ribonuclease 5 is exported from the nucleus (Pizzo *et al.*, 2013). This results in a reduction of rRNA production in the nucleolus and simultaneous break-down of tRNAs in the cytoplasm. [Interestingly, in non-neural cells when cellular stress subsides and growth resumes, Ribonuclease 5 translocates to the nucleus where it stimulates rRNA transcription; this might contribute to the anabolic phase seen in neurons recovering after chromatolysis]. There is evidence (from non-neural tissues) that RNH1 binds to ribosomes and that RNH1 deficiency leads to decreased polyribosome formation whereas overexpression of RNH1 promotes polyribosome formation (Allam *et al.*, 2017). When considered alongside the evidence described above that ribosomes also contain inactivated ribonucleases (Datta and Ghosh, 1962; Bransgrove and Cosquer, 1978; Bates *et al.*, 1985a; Bates *et al.*, 1987), it is possible that ribonucleases are activated during periods of cellular stress (e.g., axotomy) after oxidative stress relieves them of their inactivation by RNH1. Unfettered ribonucleases would then be free to degrade polyribosomes (into free monoribosomes) and monoribosomes (into ribosome fragments). However, currently, little is known about RNH1 and ribonucleases in injured neurons. An endogenous ribonuclease inhibitor is expressed in the intact brain and SCG in large excess of the levels of ribonuclease (Burton *et al.*, 1980; Bates *et al.*, 1985b) but it is not yet known whether, after CNS injury, ribonuclease net activity is increased persistently (rather than transiently, as it is after SCG injury) (Bates *et al.*, 1985a). Thus, if ribonucleases are taken up by injured neurons, they might be inactivated except where intrinsic ribonuclease inhibitors are lacking. If ribonuclease activity was increased persistently, then this could help explain atrophy and failure of long-distance axon regeneration after proximal CNS injury. In summary, a great deal more needs to be done to determine which ribonucleases contribute to chromatolysis.

Chromatolysis can involve ribophagy and reticulophagy

Work in the late 1960s and early 1970s showed evidence of fragmentation of RER and degradation of RER, polyribosomes and monoribosomes in autophagic vacuoles and dense bodies of severely chromatolytic neurons (Dixon, 1967; Matthews and Raisman, 1972) perhaps formed from membranes of fragments of RER themselves (Matthews and Raisman, 1972). Later, in the 1990s, it was shown that one can have complete destruction of polyribosomes into dust without destruction of RER (as seen after de-afferentation of the *nucleus magnocellularis* in developing chick (Rubel *et al.*, 1991)). There is recent data (from non-neural cells) showing that RNA, protein and membrane components of ribosomes and

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endoplasmic reticulum can be degraded by different mechanisms called Ribophagy and Reticulophagy, respectively (Kraft *et al.*, 2008; Cebollero *et al.*, 2012) in acidic lysosomes that contain numerous hydrolytic enzymes. Ribonuclease T2 may degrade rRNA (and ribosomes) in lysosomes during Ribophagy: it is the only ribonuclease active at acidic pH and mutations in this ribonuclease cause a lysosomal storage disorder in neurons in humans and fish (Haud *et al.*, 2011). Accordingly, Ribonuclease T2 may play a role in phagocytosis of ribosomes and endoplasmic reticulum in autophagic vacuoles during severe chromatolysis (Matthews and Raisman, 1972).

The mechanism of bulk autophagy in the nervous system can involve Autophagy-related proteins (Atg) including Atg5 and Atg7 (Hara *et al.*, 2006; Komatsu *et al.*, 2006). The molecular mechanisms of these forms of autophagy are beginning to be explored and can involve Atg1 and Atg7 (Cebollero *et al.*, 2012). An Ubiquitin protease (Ubp3) and its cofactor (Bre3) are involved in Ribophagy (but not bulk autophagy) (Kraft *et al.*, 2008). With respect to Reticulophagy, Atg-related proteins involved in this process appear to be regulated downstream of the ER stress sensors IRE1α, ATF6 and PERK (Cebollero *et al.*, 2012). An understanding of all these ER stress response mechanisms may shed light on chromatolysis. Indeed, IRE1α is a ribonuclease that is known to degrade a wide range of mRNAs in the ER in a process known as RIDD [regulated IRE1a-dependent decay; (Li *et al.*, 2013)] and there is some evidence that Ribonuclease 5 can be synthesised in response to ER stress by IRE1α (after kidney injury; (Mami *et al.*, 2016)). ER stress can induce autophagy in other cell types and it is possible that axonal or somatic ER stress is the initiator of chromatolysis (Ying *et al.*, 2014). The mechanisms of Ribophagy and Reticulophagy and their relationship to ER stress and chromatolysis need to be investigated in neurons in more detail; it is likely that these terms (stress, autophagy, chromatolysis) mean different things to different researchers and that clear definitions will be required to avoid confusion when trying to understand to what extent these processes overlap, cause one another, run in parallel or interact.

Does an axon fail to regenerate when its RNAs and ribosomes are degraded locally?

Even if an injured neuron does not become chromatolytic it is conceivable that axonal RNA and ribosomes are degraded by ribonucleases. PNS and some CNS axons synthesise proteins in their axons (Verma *et al.*, 2005; Twiss and Fainzilber, 2009) and axonal RNAs can be translated locally after injury (Gumy *et al.*, 2010); some serve as a retrograde injury signal (Twiss and Fainzilber, 2009). Axonal ribosomes often (but not always) eluded detection in the electron microscope (Bunge, 1973; Twiss and Fainzilber, 2009; Gold *et al.*, 2017): is it also possible that injury causes cleavage of RNA and/or ribosomes in axons? Local degradation of RNA and ribosomes within an injured branch might help explain why one branch of an axon fails to regenerate whereas other zones sprout collaterals, perhaps co-ordinated by mitochondria with ribosomes (Spillane *et al.*, 2013; Gold *et al.*, 2017). There is some evidence that IRE1α ribonuclease is active in neuronal processes (Hayashi *et al.*, 2007) but more remains to be done to determine whether ribonucleases constrain growth in axons.

Might attenuation of chromatolysis be therapeutically beneficial?

Neurons that do extend axons effectively after injury express a cohort of key "Regeneration-Associated Genes" (RAGs) and maintain low levels of key Regeneration-Inhibiting Genes (RIGs) (Chandran *et al.*, 2016). In contrast, injured intrinsic neurons often fail to produce adequate levels of proteins from many RAGs (Tetzlaff *et al.*, 1991). This contributes to their

regenerative failure as does the fact that CNS axon growth is restricted by cavity formation and growth-inhibitory extracellular substances including myelin-associated glycoprotein and chondroitin sulphate proteoglycans. To date, many methods for promoting PNS or CNS axon regeneration have focused manipulation of one or a small number of genes. Some strategies have achieved regeneration of axons in the PNS and CNS by overexpressing a single RAG (e.g., KLF7; (Moore *et al.*, 2009)) or reducing levels of a RIG (e.g., PTEN; (Jin *et al.*, 2015)).

However, some severely injured neurons undergo persistent chromatolysis and atrophy. Perhaps in severely injured neurons, this strategy of overexpressing one or a small number of genes is unlikely to succeed unless those genes can prevent the collapse of (or induce the restitution of) much of the protein synthesis machinery. Might this be feasible? Chromatolysis generally takes a few days to reach a maximum even when injury is within a few millimetres of the cell body (Matthews and Raisman, 1972). This indicates that early intervention after injury may be possible to prevent collapse of this part of the protein synthesis machinery which may lead to a more favourable outcome. Alternatively, ribonucleases might cause irreversible cleavage of RNA and ribosomes within hours of injury but the diffusion or dispersion of Nissl substance might take longer. Chromatolytic extrinsic neurons often revert to a more-normal phenotype many weeks after injury (Matthews and Raisman, 1972; Johnson and Sears, 2013). In cultured neurons and organotypic slices, fission of endoplasmic reticulum in dendrites can be followed by fusion (*i.e.*, it is reversible): it does not affect neuronal survival (Kucharz *et al.*, 2009; Kucharz *et al.*, 2013). Chromatolysis is also reversible in CNS neurons. For example, after thoracic rubrospinal tract injury, red nucleus neurons undergo mild chromatolysis that is reversed with time whereas this is largely not the case after cervical rubrospinal tract injury (Egan *et al.*, 1977b). Various treatments have also been shown to prevent or reverse atrophy in CNS neurons including neurotrophin treatment (Kobayashi *et al.*, 1997; Kwon *et al.*, 2007) and chondroitinase ABC (Carter *et al.*, 2008; Carter *et al.*, 2011) even after long delays (Kwon *et al.*, 2002). Empirical evidence is needed to determine whether blocking the chromatolytic response leads to cell death or whether it can accelerate axon regeneration. Downregulation, subcellular compartmental sequestration, inhibition or neutralisation of ribonucleases may be ways to achieve this.

ER stress can certainly arise from nervous system injury and targeting the ER stress response can increase or decrease recovery after PNS or CNS injury including spinal cord injury (Penas *et al.*, 2011; Li *et al.*, 2013; Hetz and Mollereau, 2014; Onate *et al.*, 2016). Future work may show if targeting the ER stress response can reduce fragmentation of rough endoplasmic reticulum or protect ribosomes and RNA in neurons. Although chromatolysis may have evolved as a mechanism to allow neuronal survival after injury, it may not be an optimal solution with respect to axon regeneration and with modern molecular therapies it might be possible both to maintain cell survival and accelerate the onset of axon regeneration before other factors (e.g., scar formation) intervene.

In conclusion, ribonucleases may contribute to chromatolysis, ER stress, Ribophagy and Reticulophagy after neuronal injury. Identification of which ribonucleases play deleterious role and which ribonucleases play pro-regenerative roles could be an important step in developing new therapies for repair of nervous system injuries.

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