Title: Long noncoding RNA MEG3 activates neuronal necroptosis in Alzheimer's disease

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Abstract: Neuronal cell loss is a defining feature of Alzheimer's disease (AD), but it remains unclear how neurons die and how this relates to other defining characteristics of the disease. ~~Existing in vivo AD models only partially recapitulate the neuropathology of AD with very mild or no neuronal cell loss.~~ Here we demonstrate that human neurons xenografted in mouse brain exposed to amyloid pathology develop sarkosyl-insoluble tau filaments, positive Gallyas silver staining, release phosphorylated tau (p-tau181 and p-tau231) into the blood, and display considerable neuronal cell loss, providing a model for the induction of full Tau pathology by simple exposure to amyloid pathology in AD. The alterations are specific to human neurons and contrast with the mild effects exhibited in the circumventing mouse neurons or in transplanted mouse neurons. A core transcriptional program in the human neurons is characterized by strong upregulation of *MEG3*, a neuron-specific long noncoding RNA. *MEG3* is also upregulated in neurons from AD patients in situ. *MEG3* expression alone is sufficient to induce necroptosis in human neurons in vitro. Inhibiting necroptosis using orally administered small molecule receptor-interacting protein (RIP) kinase -1 and -3 inhibitors or *RIPK3* knockout rescues neuronal cell loss in this novel AD model. Thus, xenografted human neurons in contrast to mouse neurons are uniquely sensitive to amyloid pathology, recapitulate ~~all the defining~~ neuropathological features of AD, and ultimately die by necroptosis.

One-Sentence Summary: *MEG3* activates neuronal necroptosis and inhibition of the necroptosis pathway rescues neuronal loss in Alzheimer’s disease.

Main Text: The major question of how the defining hallmarks of Alzheimer's disease (AD) amyloid-β (Aβ) plaques, neuronal tau tangles, granulovacuolar neurodegeneration and neuronal cell loss relate to each other has never been solved, largely owing to the lack of good models that encompass all neuropathological aspects of the disease. Aβ pathology strongly influences tau pathology kinetics in several mouse models, but only if tau pathology is artificially induced by extra frontotemporal dementia (FTD) causing mutations or by injecting tau-seeds isolated from AD patient brains(*1–3*). The fundamental unanswered questions remain whether amyloid-β can induce Tau pathology and how neurons die in AD.

The many attempts to generate a complete model for AD have failed likely because of (unknown) human-specific features that escape modeling in rodents. Two models have made great strides forward in using human cells to model features of AD, one reconstituting a 3D cell culture based on different types of human cells(*4*) and one using xenografted human neurons in mouse brain (*5*). We generated a much-improved mouse model for xenotransplantation of human neurons using a *Rag2*$^{-/-}$ (Rag2$^{tm1.1Cgn}$) genetic background and a single *App*$^{NL\text{-}G\text{-}F}$ (App$^{tm3.1Tcs}$/App$^{tm3.1Tcs}$) knock-in gene to drive Aβ pathology, instead of the combined *APP-PS1* (Tg(Thy1-APP*Swe, Thy1-PSEN1*L166P)21Jckr) transgene overexpression used before(*6*). Human stem cell-derived neuronal progenitor cells (NPCs) transplanted in the control *Rag2*$^{-/-}$ mice integrate well and develop over time dendritic spines ~~indicative of functionally active mature neurons~~(fig. S1A and B). Two months post-transplantation (2M PT), the xenografted neurons display characteristic mature neuronal (NEUN and MAP2) and cortical markers (CTIP2, SATB2, TBR1, and CUX2). Furthermore, unlike rodent neurons, human neurons display equal 3R and 4R tau splice forms at 6-months post-transplantation (6M PT) (fig. S1C and fig. S5H). Compared to the previously used NOD-SCID animals(*6*), *Rag2*$^{-/-}$ animals show a considerably increased life span (>18 months), allowing the study of healthy human neurons during brain aging.

We xenografted GFP-labelled H9-derived human cortical neuronal precursor cells (100,000 NPCs/mouse) into *Rag2*$^{-/-}$/*App*$^{NL\text{-}G\text{-}F}$ (further referred to as amyloid mice) or *Rag2*$^{-/-}$/*App*$^{mm/mm}$ (further referred to as control mice). β-sheet staining with dye X34 revealed robust plaque pathology in the amyloid mice (Fig. 1A & fig. S1D-E). RNA sequencing confirmed that the transcriptional profile of the human transplants at 2M in control and amyloid mice are very similar (Fig. 2A). Thus, human neurons integrate and differentiate similarly in control and amyloid mice, in line with the fact that amyloid plaque pathology appears only from 3M onwards in this model. Number of cells are also not significantly different in amyloid and non-amyloid xenografted mice at 2M (fig. S6F).

Full blown amyloid plaque pathology is seen at 18M post transplantation. At this late point in time, human neurons in the control brain appear overall healthy, display neuronal projections and dendritic spines intermingled with host microglial (IBA1) and astroglia (GFAP) cells (fig. S1D). In contrast, grafted neurons in the amyloid mice display severe dystrophic neurites near Aβ plaques, associated with microgliosis (6-7 IBA1-positive microglia per Aβ plaque) and astrogliosis

(2-3 GFAP-positive astrocytes per plaque) (Fig. 1A, and fig. S2A-B). The glial cell recruitment to plaques is very similar to non-grafted control animals (fig. S3A and B). Immunohistochemistry with phosphorylated tau (P-tau) antibodies, AT8 (P-tau Ser202, Thr205), PHF1 (P-tau Ser396 and Ser404), and MC1 (pathological conformational tau epitope) demonstrate considerable deposition of neuritic plaque tau (NP-tau)(*1*) in the human neurons of the grafted amyloid mice (Fig. 1C and D) (Surface stained around Aβ plaques (20 µm ring) was ˜2% (AT8), ˜5% (PHF1), and ˜3% (MC1), Fig. 1E-G). Interestingly, both NP-tau- and AT8-positive neurons appeared as early as 6-months post-transplantation (fig. S1E), indicating that amyloid deposition drives tau phosphorylation early on in this model. Significant tau pathology is not observed in mouse neurons in the same animal, nor in human grafted control, nor in non-grafted or mouse neurons grafted amyloid mice (Fig. 1C-D, fig. S1E, fig. S3C, H and fig. S4A-C).

Pathological, β-sheet X-34-positive tau-reactivity is seen in the xenografted human neurons (fig. S2C). Furthermore, Gallyas silver staining and tau-immunogold labeling of PHF fibril-like structures extracted with sarkosyl from grafted neurons in the amyloid mice (Fig. 1H and I) confirmed the progression of P-tau into pathological states (> 20 tau-fibrils/EM grid (control n=4, amyloid n=4) (fig. S6F). Such pathology is largely absent in grafted control or non-grafted amyloid mice (Fig. 1H, fig. S6F & fig. S3I-J). Finally, and clinically relevant, we found that p-tau181 and p-tau231(*7*, *8*) is significantly increased in the plasma of the grafted amyloid mice but not in control grafted or non-grafted mice (Fig. 1J-K), reflecting an increased secretion of soluble P-tau species from neurons into the bloodstream in response to amyloid pathology, mimicking what is observed in humans with AD.

It is possible to quantify accurately up to 0.1 ng of human DNA in a mixture with 100 ng mouse genomic DNA using qPCR(*9*) (fig. S6D)**.** Using this assay, we estimate that up to 50% fewer human neurons were present in the amyloid animals compared to control animals (Fig. 1L and fig. S6G) at 6M and 18M post transplantation. There is no significant increase in total number of human cells in control mice between 6M and 18M post transplantation (fig. S6E). The number of mouse neurons was also not significantly affected in the non-grafted control and amyloid mice (fig. S3E-G). Thus, human neurons, but not mouse neurons, develop ~~robust neuropathological A~~ AD-like tau pathology changes including p-tau and tau fibrils upon exposure to amyloid plaques *in vivo* and degenerate much like their counterparts in the human brain by an unknown death process.

Previous work has shown (opposing) effects of $Rag2^{-/-}$ immunodeficiency on amyloid plaque pathology and cellular responses(*10*, *11*). Comparison of soluble Aβ (Aβ40 & Aβ42) and guanidine-extractable Aβ (Aβ40 & Aβ42) revealed no significant differences between $Rag2^{-/-}/App^{NL\text{-}G\text{-}F}$ and $App^{NL\text{-}G\text{-}F}$ mice (not shown). Bulk RNA sequencing indicated that main aspects of the innate microglia and astroglial cellular transcriptomic responses to amyloid plaques are maintained in the $Rag2^{-/-}/App^{NL\text{-}G\text{-}F}$ immune-deficient mouse compared to a previously characterized non-immune suppressed amyloid mouse (*APP/PS1*)(*12*, *13*) (Fig. 1B) (R^2=0.81 for

differentially expressed genes). Thus, while we acknowledge that our model does not have an adaptive immune system, key features of the neuropathology of AD such as amyloid accumulation, glial responses, tau aggregation, tau phosphorylation, and neuronal cell loss, are reproduced in transplanted human neurons by simple exposure to amyloid pathology.

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Transcriptional changes in xenografted neurons reveal induction of necroptosis

We isolated the transplanted neuron-positive brain regions guided by the green fluorescent protein (GFP) marker from mice at 2-, 6-, and 18-months post-transplantation and extracted total RNA for sequencing (mean human reads ˜13.3 million) (fig. S5A-B). Mapping to mouse and human reference databases generated species-specific datasets. As expected, upregulation of the mouse genes *Axl, B2m, Cst7, Ctss, Itgax, Trem2, Tyrobp, Lyz2, Ccl3,* and *Ccl4* confirms microglial activation when exposed to amyloid in the *Rag2*$^{-/-}$*/App*$^{NL-G-F}$ mice (Fig. 1B, fig. S7A-C and Supplementary Table 2)(*13*). Human samples clustered according to genotype and age (fig. S5C). Differential expression (DE) analysis of the human grafts at two months showed only a few significantly downregulated DE genes, of unknown relevance (Fig. 2A, Supplementary Table 1). In contrast, DE of grafts in amyloid and control conditions at 6M and 18M revealed 916 up and 73 down-regulated genes, and 533 up and 34 down-regulated genes, respectively (Fig. 2B-C, and Supplementary Table 1). Gene alterations of interest include *CD74*, a marker for tau tangle-containing cells(*14*); *HBB*, a cortical pyramidal neuronal marker(*15–17*) and *A2M*, which is associated with neuritic plaques in AD human brains(*18*) (fig. S5E). In addition to the neuronal signature, in line with our previous findings(*19*), we also observe astroglial and oligodendrocytic genes, including *MOBP, MBP, OPALIN, S100A6, APOE,* among others, suggesting that the grafted NPC cells not only generate human neurons but also glia (fig. S5F). The overall expression profiles of the grafts exposed to amyloid plaques at 6M and 18M show strong correlations (R2 = 0.88 for genes differentially expressed at one or both time points), suggesting that the pathological cell states of neurons at 6M and at 18M are similar (fig. S5G).

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We investigated whether the gene signatures of the human neurons exposed to amyloid in xenografted mice are overlapping with transcriptional changes observed in human AD brains (Supplementary Table 3). We found a remarkable enrichment using gene set enrichment analysis (GSEA) between previously published AD datasets, including the ROSMAP cohort(*20*) and our data in transplanted neurons at 6M and 18M PT (P.adj <0.05) but not at 2M (fig. S8A-B). Furthermore, GSEA also revealed a striking enrichment in upregulated genes from transplanted neurons and neurons directly reprogrammed from fibroblasts of AD patients (M6: P.adj=1.64e-05, M18: P.adj=2.19e-04). These data confirm that our transplanted neurons capture AD-relevant transcriptional signatures(*21*).

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Functional gene ontology (GO) enrichment analysis of significantly upregulated genes (P.adj <0.05) at 6M using DAVID and REVIGO covered semantic space around positive regulation of transcription, protein phosphorylation, positive regulation of MAPK cascade, inflammatory responses, including TNF and interferon signaling, cell proliferation, aging, tissue regeneration,

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and myelination. (Fig. 2D and Supplementary Table 1). ~~It is well established that~~ Some reports have suggested that neurons in AD display signatures of hypo-maturity, dedifferentiation, and cell cycle re-entry(*21–24*). Using previously published datasets (Supplementary Table 1) and Gene set enrichment analysis (GSEA), we find that cellular signatures of hypo-maturity, dedifferentiation, P53, HIFα signaling, NF-kB, Myc, TGFβ-signaling, regulation of cell cycle (positive and negative), and cell-cycle re-entry are indeed enriched in the 6M and 18M grafts but not at 2M (Fig. 2E). Conversely, mature neuronal marker pathways, such as synaptic plasticity, synaptic transmission, long-term potentiation, and axon guidance were not significantly enriched (Fig. 2E).

How neurons die in AD remains a highly controversial discussion(*25*, *26*). As shown in Fig. 1L and fig. S6G, about ˜50% of xenografted human neurons are lost in the amyloid mice. We did not observe any significant alterations in the expression of genes associated with cell death mechanisms such as apoptosis or ferroptosis, but found significant upregulation of *MLKL*, the gene encoding the executor protein of necroptosis (Fig. 2B-C, fig. S2E & fig. S6A-C). This is not observed in the transcriptome of the host tissue (fig. S7A-C). We used pRIPK1 (Ser 166), pRIPK3 (Ser 227), and pMLKL (Ser 358) specific antibodies to stain brain tissue from 18M grafted and non-grafted mice. This resulted in intense punctuate staining with a vesicular pattern in the soma of the neurons in grafted amyloid mice. These vesicular structures co-stained with casein kinase 1 delta (CK1δ), a marker for granulovacuolar degeneration (Fig. 2L and fig. S6I). Non-grafted control and amyloid animals or mouse neurons derived from NPC and similarly transplanted into mice did not display these pathologies (fig. S3D and fig. S4D).

We validated this observation by qPCR on RNA extracted from the temporal gyrus of AD and age-matched control brain samples, which revealed significant upregulation of *MLKL* and *RIPK3*, an upstream kinase in the necroptosis pathway in the human brain (Fig. 2F-H). Based on initial observations made in the xenograft model described here, we have already extensively confirmed the presence of necroptosis markers in granulovacuolar degeneration in neurons of AD patients in a previous publication(*27*). Thus, our model has predictive value for the neuropathology of AD.

***MEG3* modulates the neuronal necroptosis pathway**

We identified 36 up and 21 down-regulated long noncoding RNAs (lncRNA) in the grafts of 6-month-old animals (Supplementary Table 1). Some of these non-coding RNAs (e.g., *NEAT1*) have been implicated in AD(*28*), but overall it is unclear whether and how they contribute to pathogenesis. In our data, the long non-coding RNA *MEG3* (Maternally Expressed 3) was the most strongly (˜10 fold) upregulated gene in human neurons exposed to amyloid pathology (Fig. 2B-C & fig. S2D), but not in the host mouse neurons (fig. S7D-F). *MEG3* has been linked to cell death pathways(*29*) via p53(*30*), is involved in the TGFβ pathway(*31*), and has been associated with Huntington's disease(*32*). In a cerebral ischemia-reperfusion injury model, repression of *MEG3* has beneficial effects(*33*).

While *MEG3* has not been previously implicated in AD, we found upregulation of *MEG3* in AD patient brains in a single nucleus transcriptomic database(*34*). We confirmed a 2-3-fold upregulation of *MEG3* in RNA extracted from the temporal gyrus of human AD brains (Supplementary Table 5) using qPCR (Fig. 2I). *MEG3* in situ hybridization combined with cell-specific immunohistochemical markers NeuN (neurons), IBA1 (microglia), and GFAP (astrocytes) demonstrated its exclusive expression in the nucleus of neurons (fig. S9A-B) and its strong enrichment in AD brains (2-3 puncta/nucleus in control and 8-10 puncta/nucleus in AD brain (P= <0.0001), (Fig. 2J-K). In AD, *MEG3*-expressing neuronal nuclei appeared blotched with reduced DAPI intensity, and *MEG3*-positive neurons also displayed high levels of the necroptosis marker pMLKL (fig. S9C).

MEG3 has 15 known transcriptional variants in humans(*35*). We performed Sanger sequencing on brain cDNA and found that *MEG3* transcript variant-1 (accession number NR_002766.2) is most abundantly expressed in the human adult brain. A lentivirus was used to express *MEG3* V1 in H9-derived mature cortical neurons (Fig. 3A-B). Expression of *MEG3* in the neurons resulted in a strong reduction of cell viability at 9 days after transduction compared with control-transduced human neurons (Fig. 3C). The *MEG3* induced cell loss can be rescued by the necroptosis inhibitors ponatinib, dabrafenib or necrosulfonamide (NSA) or by CRISPR mediated deletion of *RIPK1, RIPK3, and MLKL in vitro* (Fig. 3I-J and fig. S10H-J). Immunohistochemistry analysis revealed the presence of activated necroptotic markers, pRIPK1 (S166), pRIPK3 (S227), and pMLKL (S358) (Fig. 3D-F). Necroptosis-positive neurons displayed a reduced amount of cytoskeletal filament neurofilament-H (NF-H). Western blot analysis confirmed increased pRIPK1 (S166) levels (Fig. 3G), showing that the neurons were dying from necroptosis.

Next, to understand to what extent the *MEG3* expression contributes to transcriptional changes identified in the xenografted neurons, we performed RNA sequencing of *MEG3* transduced neurons 7-days post-transduction. To compare transcriptomic signatures of the *MEG3* transduced neurons with those identified in xenografted neurons, we performed gene set enrichment analysis (gene set enrichment analysis). We ranked the genes according to their log fold change differential expression between amyloid-exposed and non-exposed xenografted neurons (at M6, see Fig. 2C), and compared those to the top genes upregulated in response to *MEG3* transduction in neurons in vitro. We found a significant enrichment (NES=1.5, P.adj=1.2e-05) (Fig. 3H and Supplementary Table 4). This suggests that some transcriptional changes in the xenografted neurons might be secondary to the upregulation of *MEG3*. DE analysis of *MEG3* transduced neurons revealed no changes in necroptosis genes (fig. S10A-C) but DAVID Gene Ontology (GO) analysis of leading edge genes from GSEA on upregulated genes from 6M transplanted human neurons and *in vitro* *MEG3* expressing neurons revealed signatures of NF-kB and TNF signaling which have been related to necroptosis induction(*36*). In addition, signatures of lipid transport, interferon-γ signaling, positive regulation of peptidyl-tyrosine phosphorylation, and apolipoprotein-L signaling are observed (fig. S10D and Supplementary Table 4).

Inhibition of necroptosis by small molecules or ablation of *RIPK3* blocks human neuronal loss in the xenografted mice

We first used a previously characterized shRNA antisense construct (PMID: 31656536 and PMID: 32201430) against *MEG3* to confirm the direct role of MEG3 expression in the transplanted neurons when exposed to amyloid plaques. Downregulation of *MEG3* improved significantly the neuronal survival and was associated with downregulated expression of necrosome proteins (Fig. 3K-L and fig. S10E-G). It remains however unknown whether the neurons that degenerate during AD or in our neuronal transplants are all dying by necroptosis or whether necroptosis markers are limited to the neurons that are remaining at the stage of pathological analysis. We thus treated xenografted mice (3 groups of n=5) from 2M until 6M post-transplantation with the orally available necroptosis kinase inhibitors, ponatinib (30 mg/kg), and dabrafenib (50 mg/kg). Ponatinib inhibits RIPK1(*37*) and RIPK3(*38*) and is an FDA-approved drug for the treatment of acute lymphoid leukemia (ALL) and chronic myeloid leukemia (CML). Dabrafenib is a more specific inhibitor of RIPK3 (*39*). Immunostaining at six months revealed a striking reduction in the levels of pRIPK1, pRIPK3, and pMLKL in both treatment groups compared to untreated mice (Fig. 4A-D), without altering the glial response to amyloid (fig. S9D). ~~Excitingly, the~~ qPCR analysis of neuronal cell numbers revealed a significant increase in neurons in the treated groups compared to control group ~~up to levels seen in the grafted control mice (without amyloid)~~ (Fig. 4E). In further experiments we have transplanted *RIPK1* or *RIPK3* knocout human stem cells generated using CRISPR/Cas9 similar to the ones used in the for the in vitro experiments above. In contrast to the in vitro experiments none of the neurons transduced with guide RNA against RIPK1 survived, indicating that knock out of RIPK1 is lethal in vivo. Previous work has indeed shown that constitutive deletion of RIPK1 in mice leads to embryonic lethality (PMID: 27819682). In contrast, deletion of the *RIPK3* gene with gRNA significantly improved neuronal survival (**Fig. 4 F-H**). Immunofluorescence staining confirmed the significant decrease of necroptosome signals in *RIPK3* deficient human neurons.

Conclusion

Our results demonstrate that amyloid pathology is sufficient to induce ~~full~~ hallmark neuropathological features of AD in non-genetically manipulated human neurons, including dystrophic neurites, tau fibrils, granulovacuolar degeneration, cell loss, and the appearance of the p-tau181 and p-tau231 biomarker in the plasma of the grafted mice. One of the major unsolved questions in AD research is how neurons die. Some reports have proposed apoptotic mechanisms, but it has never been demonstrated convincingly that this drives neuronal cell loss in the disease(*40*). While previous publications suggested activated necroptosis in the AD brain(*27*, *41*), we demonstrate here that ~~a large part of the degenerating~~ necroptosis mediated neuronal loss in the xenograft model can be rescued by treatment with clinically relevant necroptosis inhibitors or by ablating the RIPK3 gene in the transplanted neurons(*39*). Thus, we suggest that neuronal death in AD is largely driven by necroptosis, linking the loss of neurons to inflammatory processes that are upstream of this well-studied death pathway(*13*, *42*, *43*). Therefore, therapies that prevent neuronal cell loss, in combination with more mainstream Aβ and tau targeted interventions, might be useful additions to the current efforts to develop disease-modifying strategies for AD(*44–46*). Our data suggest that necroptosis is downstream of the accumulation of pathological tau and is induced by the upregulation of the non-coding RNA *MEG3* possibly via TNF-inflammatory pathway signaling. Long non-coding RNAs (lncRNAs) are important regulators of gene expression and influence a variety of biological processes, including brain aging and neurodegenerative disease(*47*). The large number of non-coding RNAs that are differentially expressed in our novel AD model warrant further investigation.

It is deeply intriguing that human transplanted neurons display an AD phenotype, while mouse neurons interspersed within the graft or NPCs derived from mouse neurons transplanted in a similar way as their human counterparts, do not display such signs. This strongly suggests that as yet unknown human-specific features define the sensitivity of the neurons to amyloid pathology. We notice that transcriptional analysis of the transplanted human neurons indicates signatures of downregulation of mature neuronal properties and upregulation of immature signaling pathways. This vibes with observations in a recent study that analyzed the transcriptional profiles in neurons directly derived from fibroblasts of AD patients (iNs)(*21*). The neurons in that study did not show tangles, amyloid, or necroptosis markers typical of AD, and it was not clear how those observations relate to the classical hallmarks of AD. While the contribution of these immature signatures to the disease process in our model needs further investigation, ~~Our data suggest alternatively, that the relative immature profile of the xenografted human neurons in our model makes them sensitive to amyloid pathology, resulting in tau pathology and other hallmarks of AD. Paradoxically, we might have mimicked in our model the reactivation of immature and progenitor-like signaling pathways or DNA repair programs, also observed in neurons from patients at risk of AD(*21*, *48*), pointing to this age associated hypo-mature neuronal identity as an important factor in the development of full blown AD pathology.~~ our data however indicate that necroptosis is an important contributor to cell death in Alzheimer's Disease. Necroptosis is an active area of drug development in cancer

(ref) and ALS (ref), and we therefore suggest that it is worthwhile to explore this pathway further for potential drug development in AD as well.

Finally, it is of crucial importance to better understand what precisely makes that transplanted human neurons become diseased when exposed to amyloid pathology, while mouse neurons remain preserved. Understanding the resilience of mouse neurons to amyloid pathology will not only help to model the disease better, but opens the door towards pathways that might protect the human brain against neurodegeneration.

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5 **Data and materials availability:** RNA sequencing data is deposited in the GEO. GEO accession number: GSE195458.

Supplementary Materials
Materials and Methods
10 Fig S1 – S10
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