RESEARCH ARTICLE

Alpha-synuclein fibrils recruit TBK1 and OPTN to lysosomal damage sites and induce autophagy in microglial cells

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ABSTRACT

Autophagic dysfunction and protein aggregation have been linked to several neurodegenerative disorders, but the exact mechanisms and causal connections are not clear and most previous work was done in neurons and not in microglial cells. Here, we report that exogenous fibrillary, but not monomeric, alpha-synuclein (AS, also known as SNCA) induces autophagy in microglial cells. We extensively studied the dynamics of this response using both live-cell imaging and correlative light-electron microscopy (CLEM), and found that it correlates with lysosomal damage and is characterised by the recruitment of the selective autophagy-associated proteins TANas it coelyen

that the TBK1–OPTN axis targets damaged mitochondria for degradation via PINK1/parkin-mediated mitophagy (Moore and Holzbaur, 2016). As an upstream binding partner for the autophagy receptor, TBK1 phosphorylates OPTN on damaged mitochondria, leading to the formation of a TBK1–OPTN complex. Inhibition and depletion of TBK1 or OPTN blocks the efficient turnover of depolarised mitochondria. Interestingly, mutations of OPTN and TBK1 are both associated with neurodegenerative diseases including amyotrophic lateral sclerosis (ALS), Huntington's disease, Alzheimer's disease, Parkinson's disease, Creutzfeld–Jacob disease and Pick's disease (Korac et al., 2013; Li et al., 2016). However, the mechanistic basis underlying the specific interaction between OPTN and TBK1 in281.l2e59is1.5(eo)-(tingl) Tr-29y

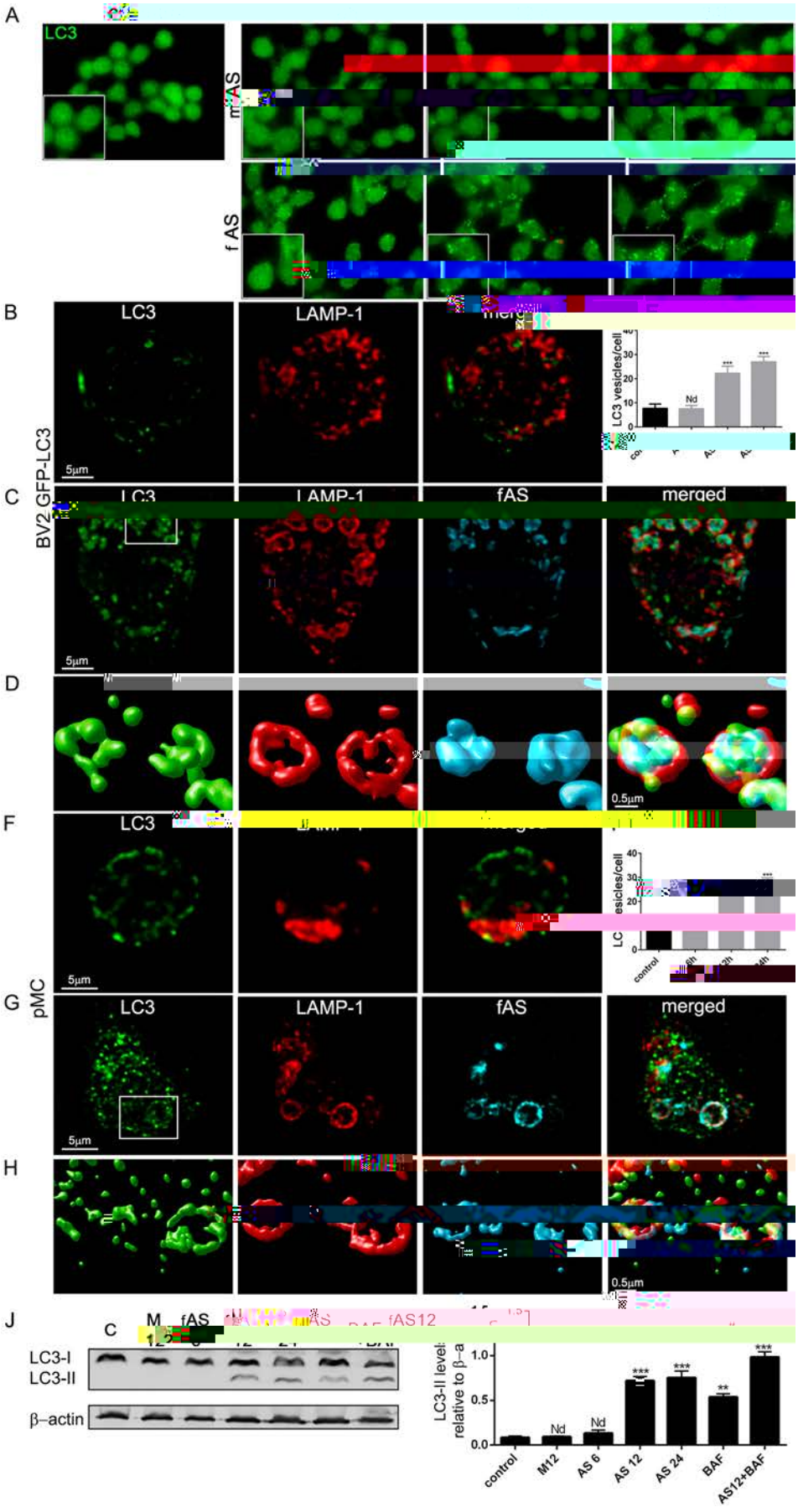

Fig. 1. Alpha-synuclein induces autophagy in microglial cells. (A) BV2 microglial cells were left untreated or stimulated at different time points with AS monomers (mAS) or fibrils (fAS) at 1 μM. Cells were then fixed and stained for LC3. (B–H) BV2 GFP–LC3 cells (B,C) or primary microglial cells (F,G) were left untreated or stimulated with Alexa Fluor 647-labelled AS fibrils (1 μM). After 12 h cells were immunostained with anti-LAMP1 (red) antibody and primary microglial cells were also stained for LC3. Images shown are *z*-stack projections. (D,H) 3D surface-rendered magnifications of the selected area above. (E,I) Mean±s.e.m. LC3-positive vesicles in unstimulated or treated BV2 (E) and primary microglial cells (I) were determined using ImageJ particle counting

and it induces autophagy in microglial cells. The monomeric conformation was not able to activate the autophagy pathway at the time and dose studied.

Autophagy dynamics of AS-stimulated microglial cells

To further study the autophagic response triggered by fAS in microglial cells, we conducted live-imaging experiments at different time points after fAS stimulation. We used BV2 GFP–LC3 microglial cells and LysoTracker Blue for lysosomal staining. We did not observe a significant increase in LC3 puncta during the first 8 h after fAS stimulation (Fig. S1B–D, Fig. S2A–C; Movies 1–4). Of note, fAS was quickly internalised during the first 20 min by microglial cells and showed lysosomal localisation from the earliest time points (Fig. S1B–D; Movie 5).

Interestingly, after 12 h of stimulation we detected a substantial increase in the autophagy response. LC3 vesicles increased over time and were predominantly associated with LysoTracker+/fAS+ vesicles, forming a ring-like structure around them, as observed previously using confocal analysis (Fig. 2A,B; Fig. S2C; Movies 6, 7). In additional experiments, BV2 cells stably expressing GFP–ATG13 were co-transfected with a CFP–LC3 plasmid. ATG13 integrates the autophagy initiation complex ULK1, the most upstream complex of the autophagy pathway and is essential for autophagosome formation (Axe et al., 2008; Karanasios and Ktistakis, 2015). In agreement with previous reports and autophagy dynamics studies (Ktistakis et al., 2014), we observed positive ATG13 signal as an early event during autophagosome formation, closely associated with LC3 puncta, and its lifetime was shorter than the same structures containing LC3 (Fig. 2C,D; Fig. S2C; Movies 8, 9). As seen before, the LC3 signal associated with ATG13 progressed into characteristic rounded structures around synuclein fibrils (Fig. 2C,D).

Taken together, these results indicate that the autophagic response to fAS follows a canonical route (utilising ATG13-positive structures that mature into LC3-positive structures), but it is not an immediate event after synuclein treatment. The fact that lysosomes containing fAS are surrounded by LC3 vesicles suggests the possibility that the autophagic machinery may respond to lysosomal damage caused by the fibrils, and this is a question we will address later.

CLEM study of fAS-stimulated microglial cells reveals canonical autophagy

There are increasing numbers of reports showing the involvement of the non-canonical autophagy pathway in diverse pathological conditions since it was described for the first time. Although important advances have been made in the molecular characterisation and differentiation between these alternative routes, we are still far from precisely understanding the mechanistic details and limits of both pathways (Dupont et al., 2017; Martinez et al., 2015). The principal difference between autophagosomes and non-canonical vacuoles is that the former have two limiting membranes positive for LC3, whereas the latter have one. In order to discriminate these different processes, we conducted CLEM experiments and analysed the presence of single- or double-membrane LC3-positive vesicles after fAS stimulation of BV2 GFP–LC3 microglial cells.

We clearly detected double-membrane autophagic vesicles (AV) mainly correlating with GFP–LC3 signal and closely associated with fAS+ structures (Fig. 3A–F; Movies 10–12). Furthermore, we also observed double-membrane vesicles and multi-membrane structures surrounded by a single-limiting membrane, probably as a result of fusion events between autophagosomes and lysosomes (Fig. 3A–F; Movies 10–12). These results are in agreement with a previous report describing similar AVs found on neocortical biopsies of human brain from individuals with Alzheimer's disease (Nixon et al., 2005). In agreement with Nixon et al., the morphologies and composition of vesicles that accumulated after fAS treatment corresponded to those of the vesicular compartments

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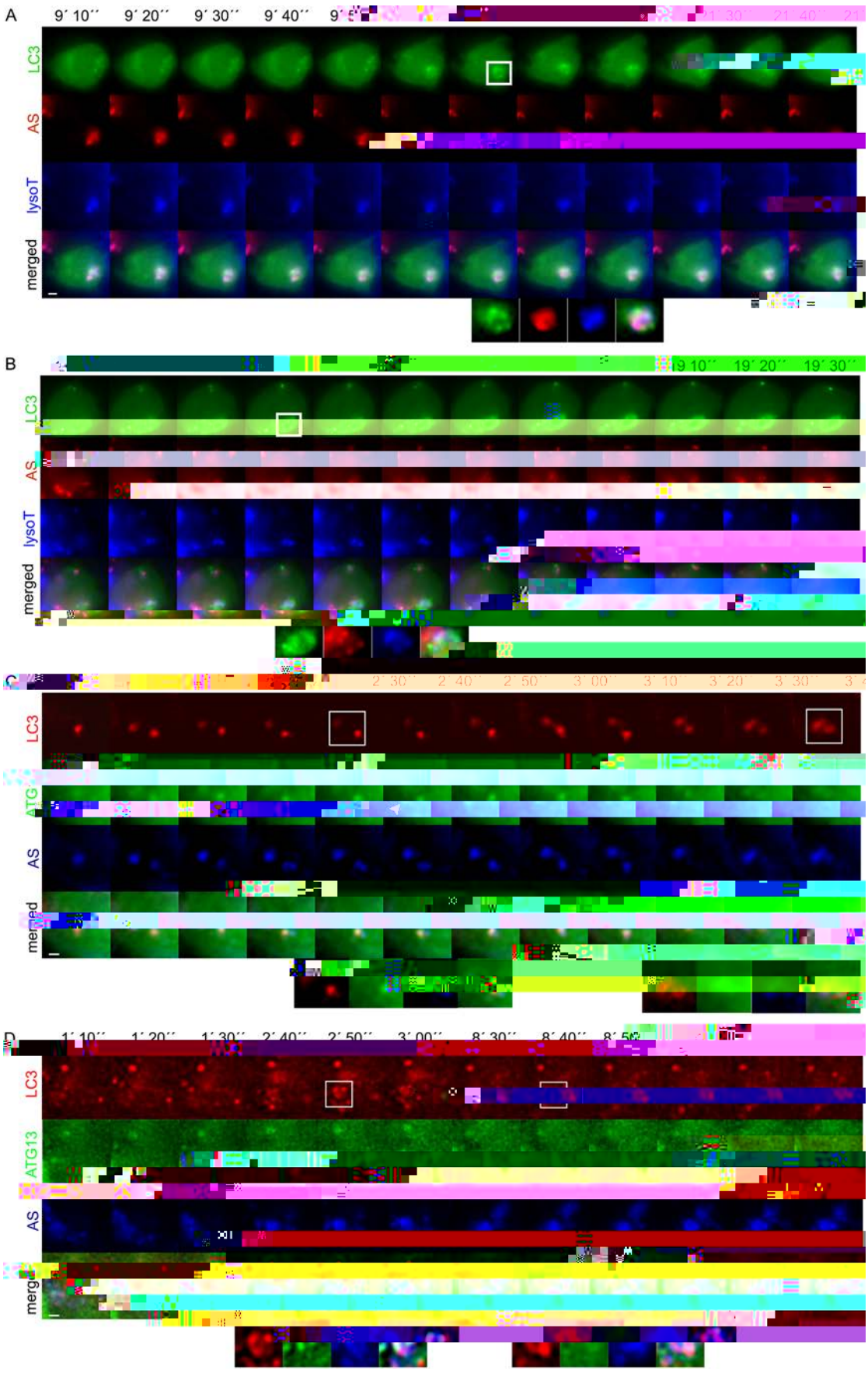

Fig. 2. See next page for legend.

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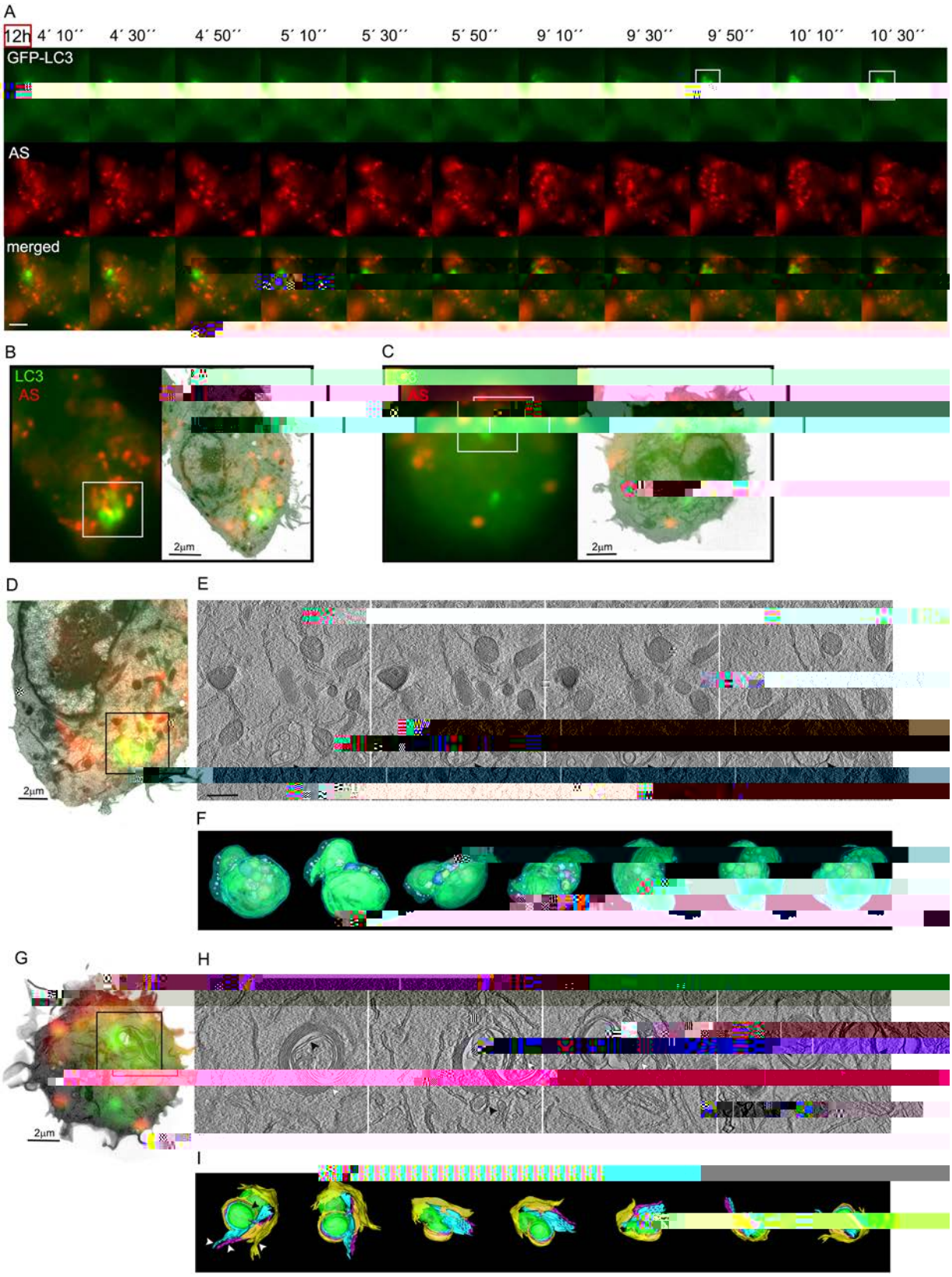

Fig. 4. See next page for legend.

These results indicate that lysosomes respond to the presence of fAS increasing cathepsin-B activity, which decreases with the progression of lysosomal damage.

In parallel experiments, we also evaluated mitochondrial status, as mitochondrial dysfunction has been associated with several neurodegenerative diseases and AS has been shown to alter

mitochondrial activity (Esteves et al., 2011). However, we did not find changes either in mitochondrial mass nor mitochondrial cell membrane potential after fAS stimulation at the time points studied (Fig. 5I). Overall these findings indicate that fAS induces lysosomal but not mitochondrial damage, with a similar kinetic to that observed in autophagy activation, suggesting that this response is concomitant to lysosomal impairment.

TBK1 and OPTN are recruited to damaged lysosomes in AS-stimulated microglial cells

Previous studies performed in *Salmonella enterica*-infected HeLa cells have shown that TBK1 directly phosphorylates the autophagic adaptor OPTN and that this interaction allows the autophagic machinery to be recruited to the intracellular loci of the bacteria, resulting in elimination of the bacteria by lysosomes. Furthermore, OPTN has been observed in protein inclusions of various neurodegenerative diseases, such as ALS and PD. Thus, we aimed to determine whether TBK1 and OPTN could be recruited to damaged lysosomes in AS-stimulated microglial cells.

As the autophagy response was triggered at a similar time point to that described for AS-induced lysosomal damage (Fig. 5C–G, 12 h time point), we aimed to determine whether microglial autophagy could be activated as a means to control lysosomal quality rather than by AS internalisation itself. Therefore, we analysed the dynamics of TBK1 and OPTN activation in AS-stimulated microglial cells using immunoblot and confocal immunofluorescence.

Interestingly, we found that levels of TBK1 and OPTN phosphorylation were significantly increased after 12 h of fAS stimulation but not during earlier time points or after AS monomeric stimulation (Fig. 6A,B).

In parallel experiments, we observed using confocal microscopy that TBK1 and OPTN co-localise with ubiquitin and Gal-3 puncta in AS-stimulated BV2 and primary microglial cells (Fig. 6C; Fig. S3), indicating the recruitment of both proteins to ubiquitylated lysosomal damage sites. In accordance with our previous results (Fig. 6A, Fig. 5), TBK1 and OPTN puncta formation were only observed after 12 h of fAS stimulation, which contrasted with the diffuse staining pattern observed in the control conditions (Fig. S3A,B). Moreover, we also observed LC3- and AS-positive vesicles co-localizing with both TBK1 and OPTN (Fig. 6D).

In additional experiments, we used confocal immunofluorescence to analyse whether the autophagy receptors p62 and NDP52 would also be recruited to fAS-positive vesicles. Although mouse NDP52 is a truncated form lacking the C-terminal zinc-finger domain that interacts with ubiquitin, it has been shown to bind phosphorylated TAU via the SKICH domain and to facilitate autophagy-mediated degradation of TAU in mouse (Jo et al., 2014; Minowa-Nozawa et al., 2017), indicating that NDP52 recognises different targets through different domains. Nonetheless, we did not observe co-localisation of either NDP52 or p62 with fAS after 12 h of stimulation (Fig. S4A,F). As expected, NDP52 did not co-localise with ubiquitin in fAS-stimulated microglial cells (Fig. S4C). Moreover, we did not find an increase in p62 puncta formation after fAS stimulation in microglial cells (Fig. S4D,E), suggesting these adaptors do not function together in targeting fAS-containing organelles.

Taken together, these results show that TBK1 and OPTN activation and recruitment to damaged lysosomes displays a similar kinetic to that described for autophagy induction (Figs 1, 2), suggesting both proteins participate in the autophagic turnover of this organelle and that microglial autophagy is activated as a response to AS-induced lysosomal impairment.

TBK1 inhibition impairs LC3 recruitment to damaged lysosomes

In a previous report, Moore and Holzbaur (2016) showed that TBK1 and OPTN are recruited to mitochondria after acute damage, and that loss or chemical inhibition of TBK1 disrupts mitophagy. We next aimed to determine whether TBK1 inhibition would affect autophagy adaptor recruitment and LC3 targeting of damaged lysosomes in fAS-stimulated microglial cells. Firstly, we evaluated the effectiveness of BX795, MRT67307 and Amlexanox, three chemical inhibitors of TBK1. As shown in Fig. 7A, all three

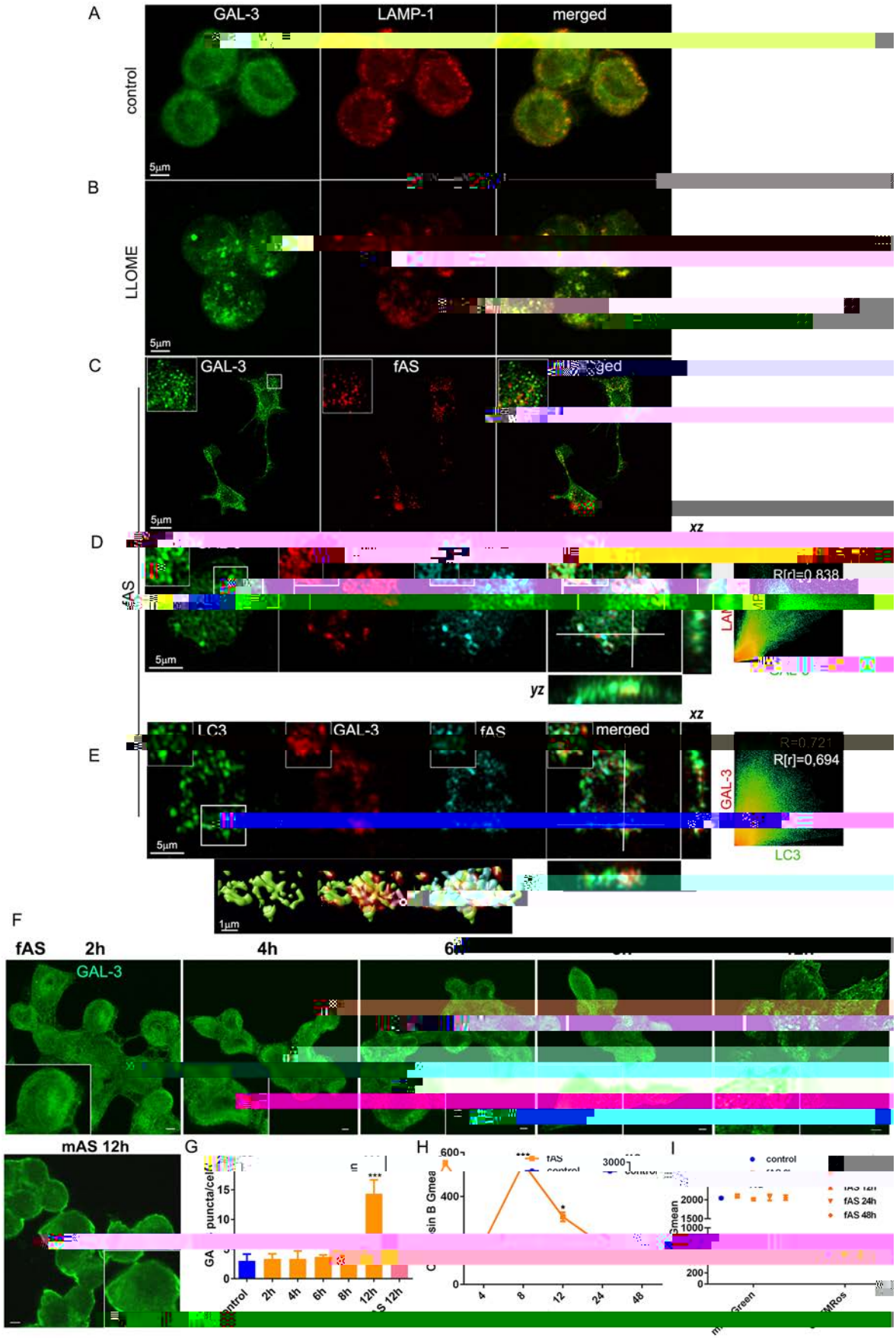

Fig. 5. See next page for legend.

protein required for autophagy induction and autophagosome formation (Hara et al., 2008). We evaluated the ability of spautin-1 and FIP200 siRNA to inhibit autophagy, using confocal microscopy analysis of LC3 puncta formation after treating BV2 GFP–LC3 microglial cells with PP242, a specific mTORC1 inhibitor and autophagy inducer. We observed that both treatments significantly suppressed the autophagy response (Fig. S5A,B). Moreover, autophagy blockade by spautin-1 and FIP200 siRNA also decreased LC3 puncta formation in fAS-stimulated primary microglial cells (Fig. S5C).

We next investigated whether autophagy inhibition prior to fAS stimulation affects microglial cell viability. BV2 and primary microglial cells were cultured in the presence or the absence of spautin-1 or FIP200 siRNA and stimulated with fAS for 24 h, then we evaluated microglial cell death using flow cytometry. We found an increase in the frequency of dead cells (posit(pob/T19(in)-019(in)urau2spin(ogl(bl54-y)20.62spJbnialaV)18o6(ogliw,)-2620hether autopha1

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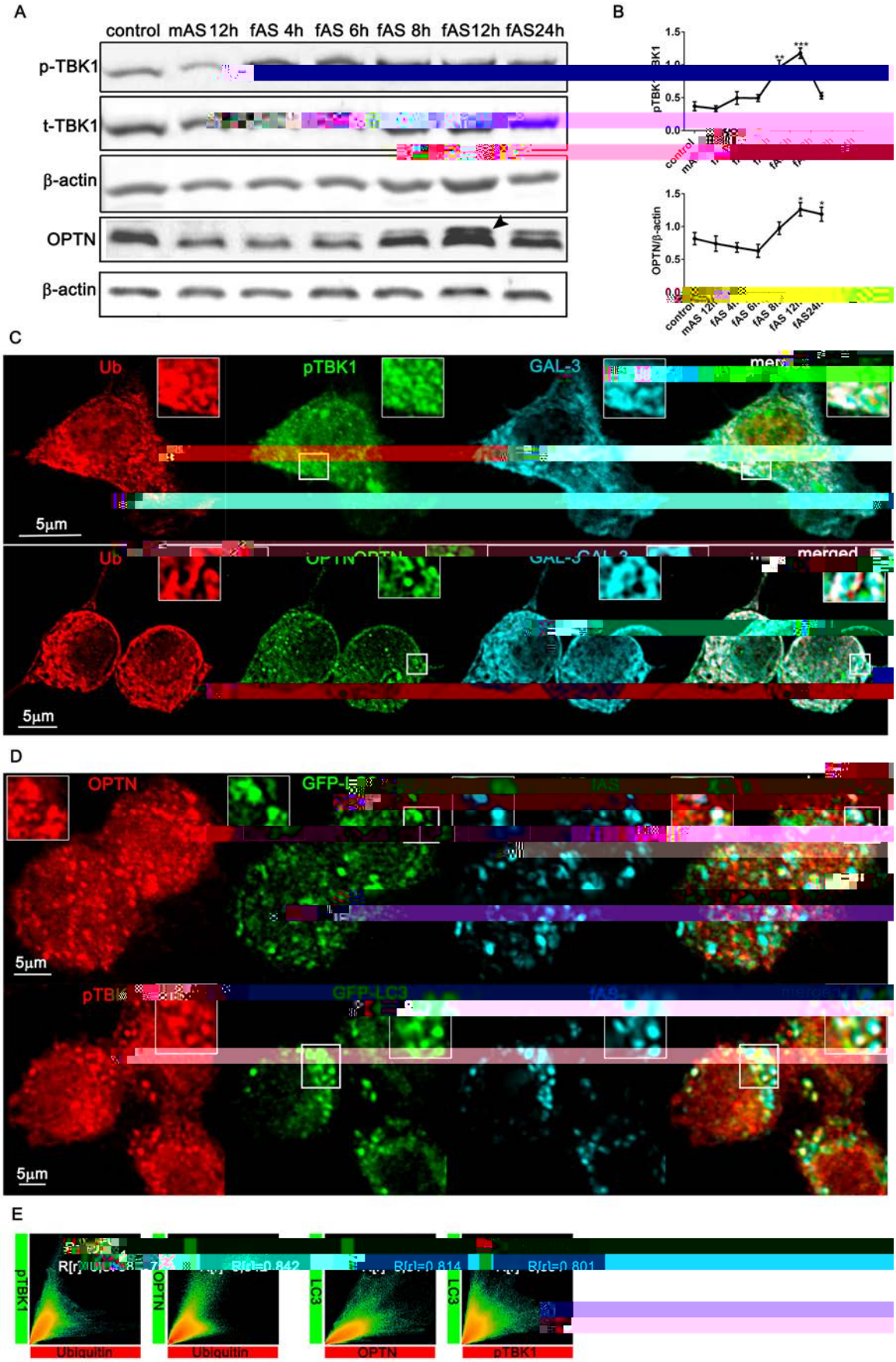

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Recent evidence indicates that AS could disturb neuronal metabolism through inducing lysosomal and mitochondrial damage (Bourdenx et al., 2014; Freeman et al., 2013; Xilouri et al., 2016). Nevertheless, the effects of AS on glial organelles is poorly understood. Here, we report that although fAS is rapidly incorporated into lysosomes after cellular internalisation, it induces lysosomal damage and TBK1/OPTN recruitment at the same time point as when the autophagy response is substantially activated (Figs 5, 6; Fig. S3). Moreover, we discovered a high degree of co-localisation between LC3 and GAL-3 puncta, and between LC3 and TBK1 and OPTN puncta, suggesting that lysosomal damage rather than fAS acts as an activator signal for autophagy induction. Importantly, we observed that TBK1 inhibition disrupts OPTN and LC3 recruitment to damaged lysosomes in fAS-stimulated microglial cells, but it did not affect autophagy induced by inhibition of mTORC1. Collectively, these results suggest that OPTN is recruited to lysosomes damaged by persistent fAS accumulation, following recognition of OPTN by TBK1 and phosphorylation of TBK1, which may allow subsequent recruitment of LC3 and the autophagy machinery as a cellular attempt at restoring lysosomal quality control.

Along this line of evidence, Flavin et al. (2017) observed, using

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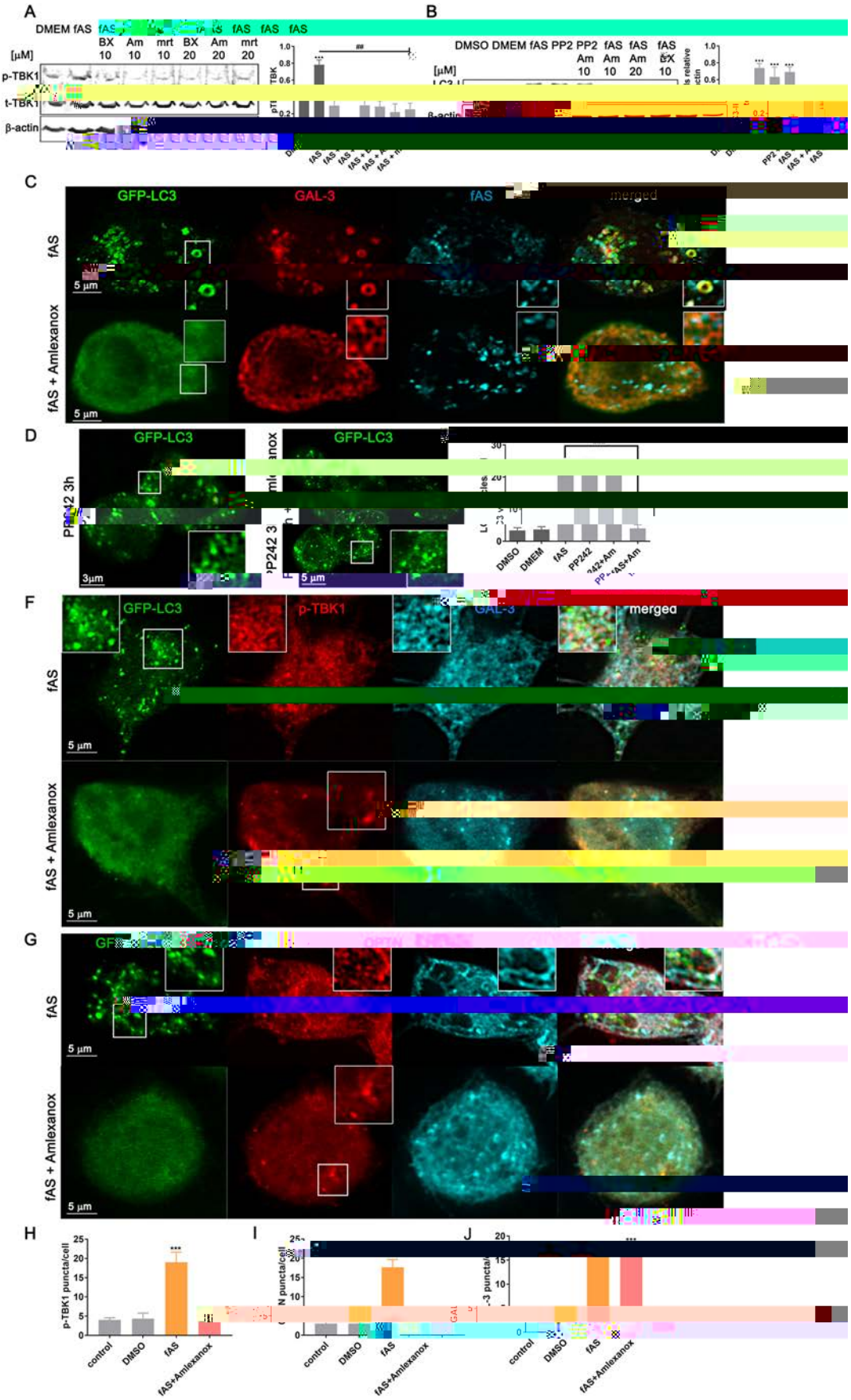

Fig. 7. See next page for legend.

aggregates and their crosstalk with the autophagy pathway not only in neurons, but also in glial cells, would shed light on novel therapeutic targets for neurodegenerative disorders.

MATERIALS AND METHODS

Reagents

Dulbecco's Modified Eagle Medium (DMEM), Opti-MEM, fetal bovine serum (FBS), penicillin, glutamine, G418, streptomycin, Silencer select FIP200 siRNA (4390771), Silencer select negative control 1 (4390843), Lipofectamine RNAiMAX (13778100) and LysoTracker Blue (L7525) were obtained from Thermo Fisher Scientific. Antibodies used for western blotting were rabbit polyclonal anti-LC3 (Sigma, L7543; 1:1000), mouse monoclonal anti-β-actin (Cell Signaling Technology, 8H10D10; 1:1000), rabbit polyclonal anti-Optineurin (Cayman, 100000; 1:1000), mouse monoclonal anti-TBK1 (Santa Cruz, 398366; 1:1000) and rabbit monoclonal anti-phosphoTBK1 (Cell Signaling Technology, 5483S; 1:1000). Antibodies used for immunofluorescence were rabbit monoclonal anti-LC3 A/B (Cell Signaling Technology, D3U4C; 1:100), rat monoclonal anti-LAMP-1 (BioLegend, 121601; 1:200), mouse monoclonal anti-galectin-3 (BioLegend, 125401; 1:300), rabbit polyclonal anti-GALNS (GeneTex, 110237; 1:100), rabbit polyclonal anti-Optineurin (Cayman, 100000; 1:100), mouse monoclonal anti-TBK1 (Santa Cruz, 398366; 1:100) and mouse monoclonal anti-Ubiquitin (Cell Signaling Technology, 3936S; 1:100). PP242 (13643) and BafA1 (11038) were purchased from Cayman. Spautin-1 (SML0440) was purchased from Sigma. MRT67307 (19916) and Amlexanox (14181) were purchased from Cayman. BX795 (HY-10514) was purchased from MedChemExpress. MitoSpy Green FM and MitoSpy Orange CMTMRos and Mitoha ig10)lS916) a

glutaraldehyde (GA, Electron Microscopy Sciences) in 0.1 M cacodylate buffer immediately after detecting the event of interest. The subsequent EM

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