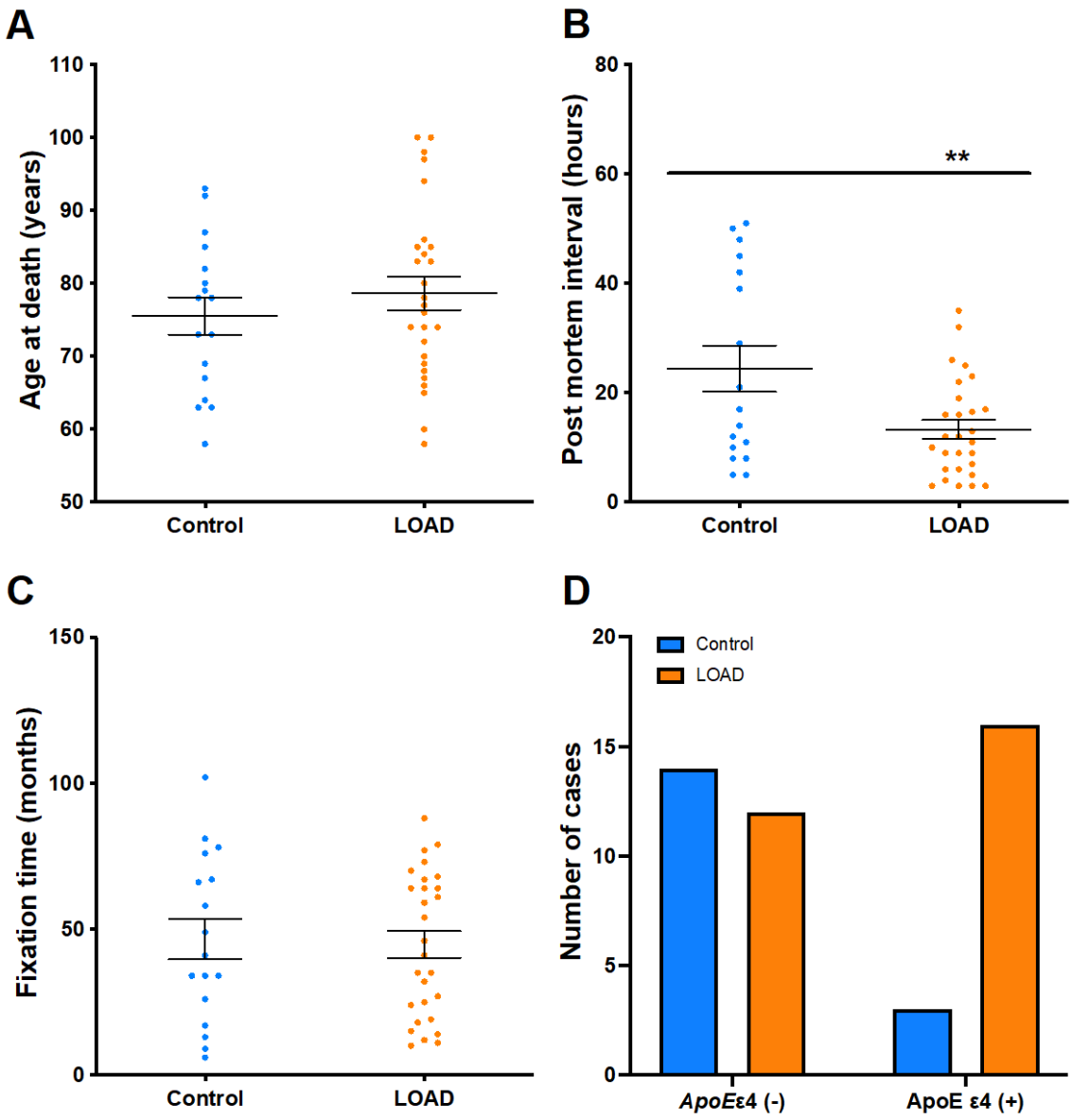
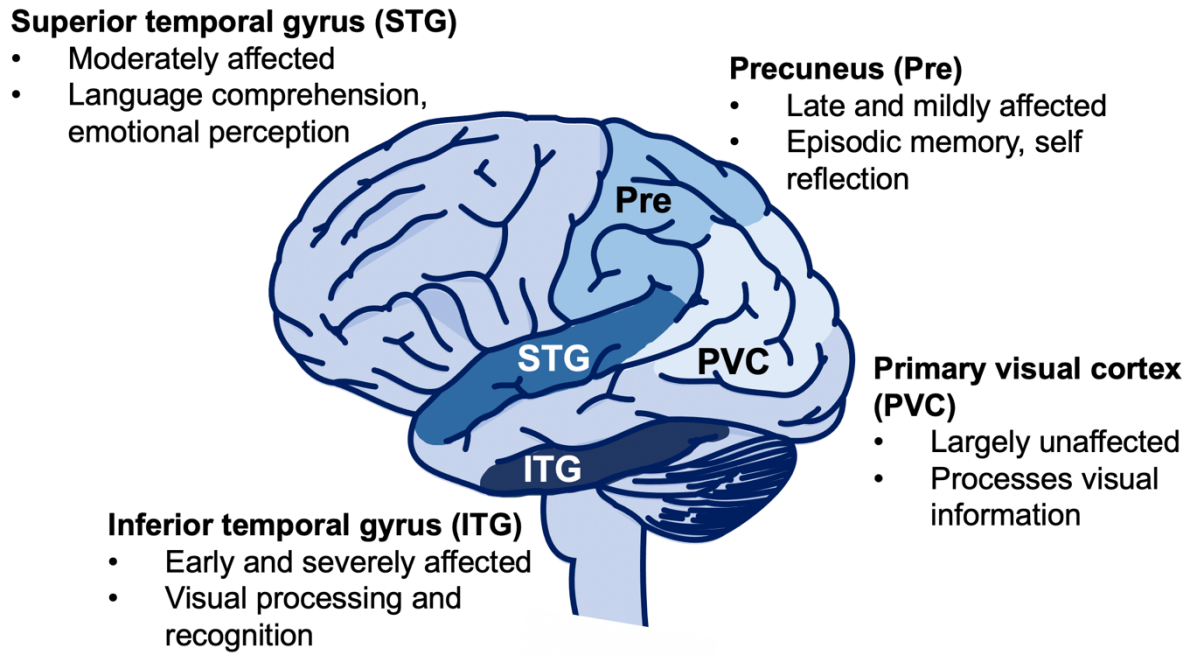
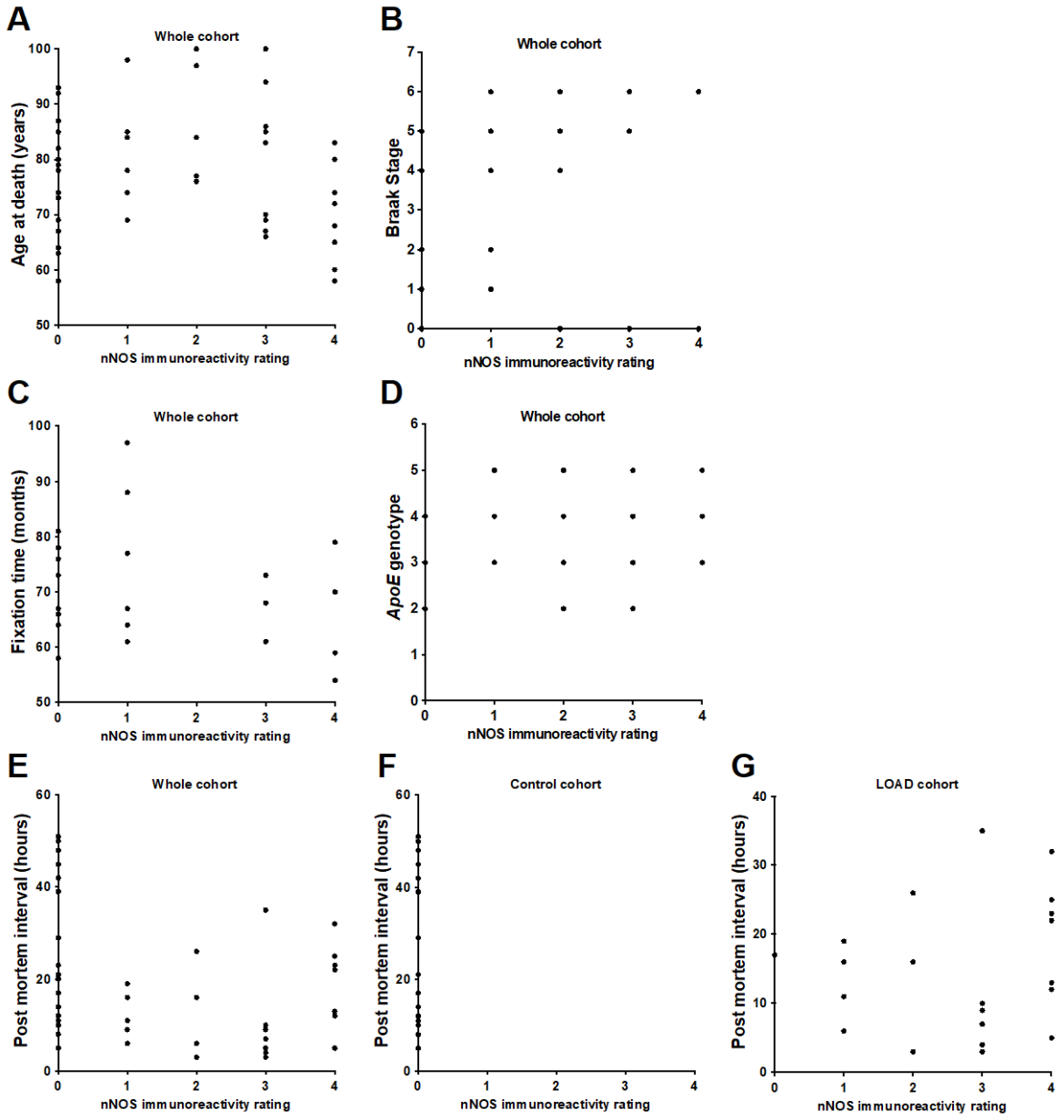
Supplementary figures



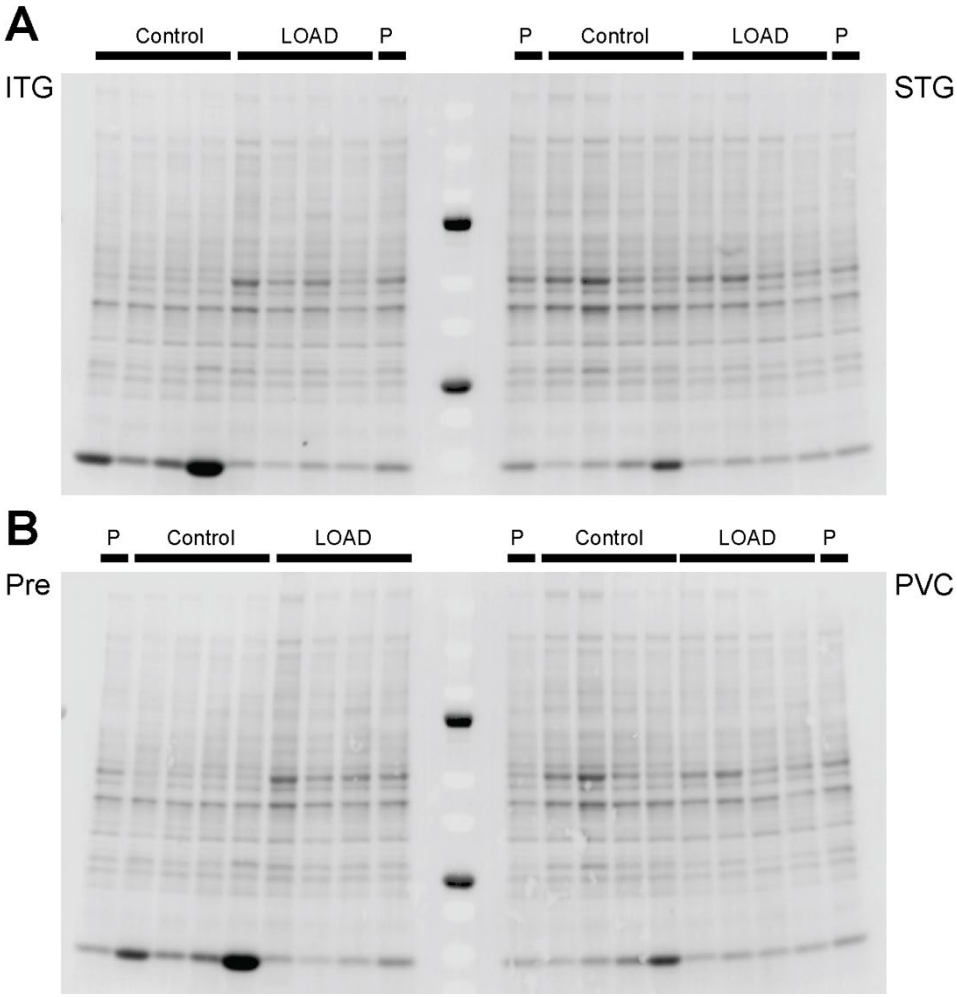
Supplementary Figure 1. Analysis of demographic and clinical indices for human *post-mortem* tissue cohort. Mean (±SEM) of age at death (years) (A), *post-mortem* interval (hours) (B), tissue fixation time (months) (C) and *APOE* genotype (D) of control cohort (blue) and late-onset Alzheimer's disease (LOAD) cohort (orange). Data points represent one case with n = 17 for control and n = 27 for LOAD. Analysis is either unpaired two-tailed Student's t test with Welch's correction (A-C) or Chi square test (D) with significant differences indicated by ** $p < 0.01$.



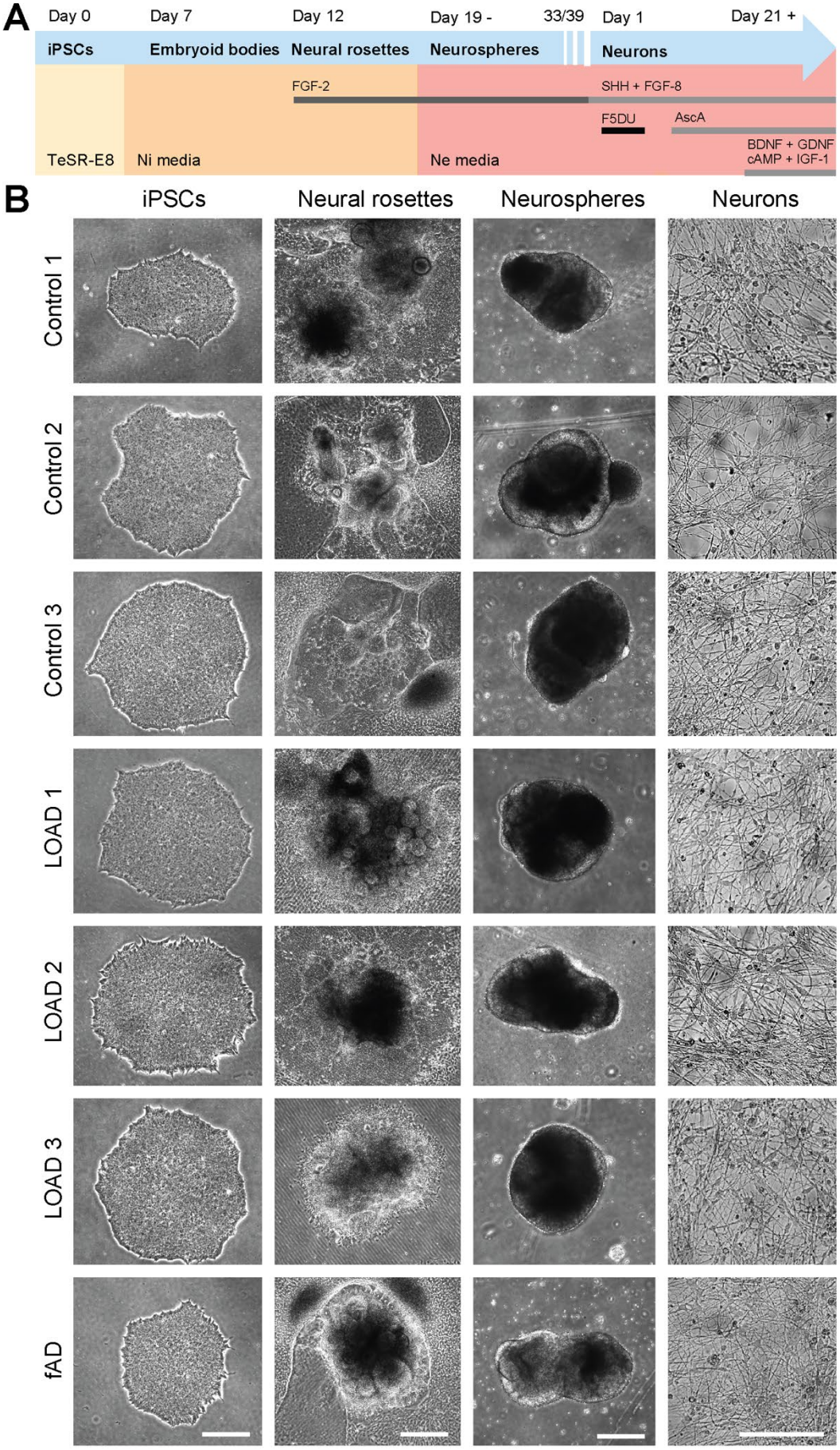
Supplementary Figure 2. Schematic of brain regions analysed for neuronal nitric oxide synthase expression in human *post-mortem* tissue from cognitively healthy (control) and late onset Alzheimer's disease patients.



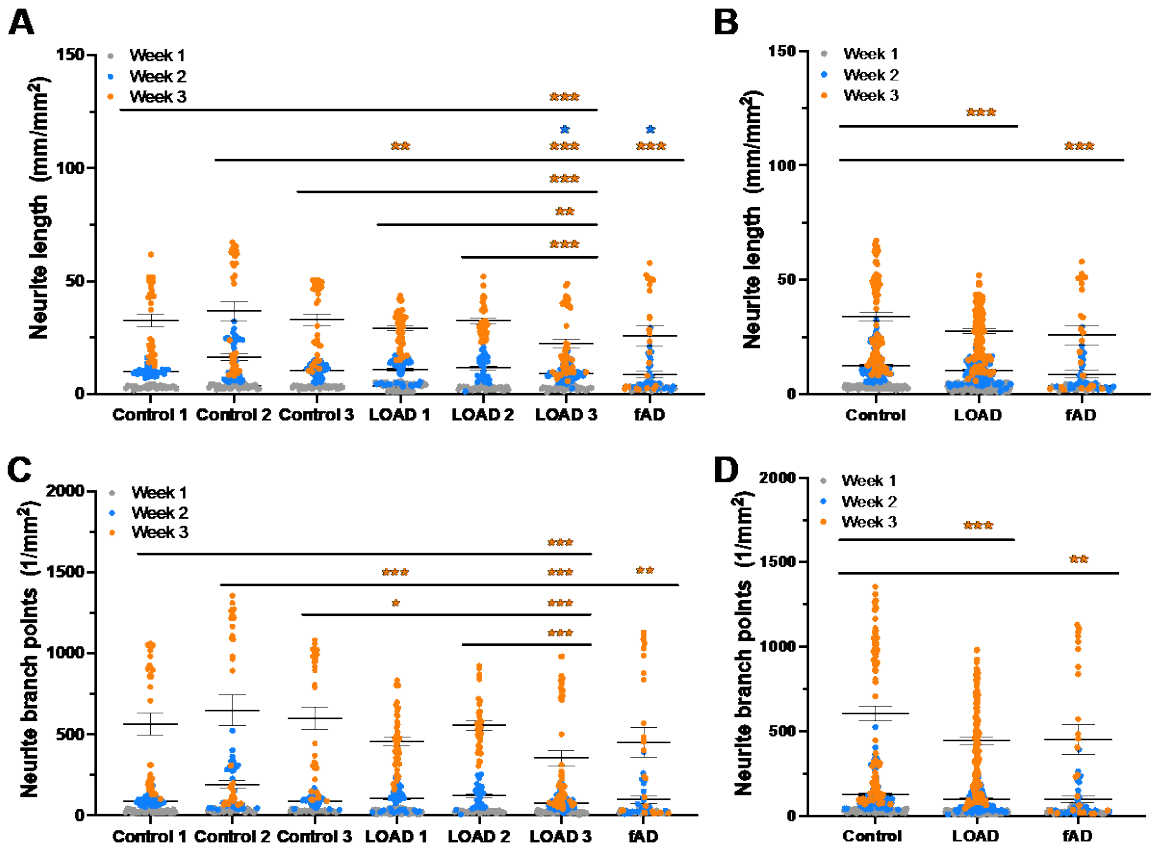
Supplementary Figure 3. Correlation of neuronal nitric oxide synthase severity with demographic and clinical indices in human *post-mortem* tissue cohort. Correlation between neuronal nitric oxide synthase (nNOS) immunoreactivity rating and age at death (years) (A), Braak stage (B), fixation time (months) (C), *APOE* genotype (D) and *post-mortem* interval (hours) (E) across the whole cohort, in addition to *post-mortem* interval (hours) in the control cohort (F) and late-onset (LOAD) cohort (G) of human *post-mortem* tissue. Data points represent one case with n = 17 for control and n = 27 for LOAD. Analysis is Pearson's r with 95% confidence interval.



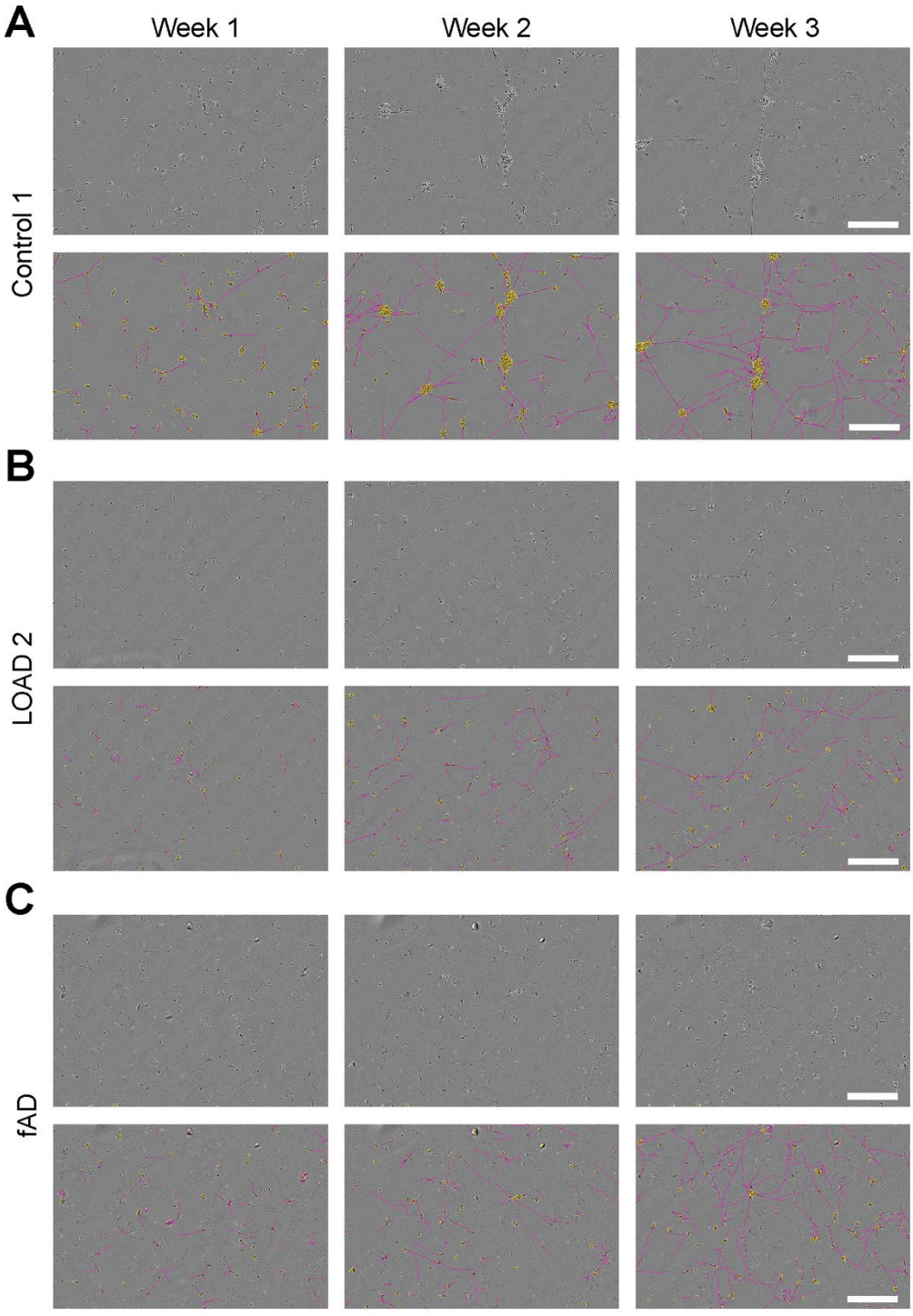
Supplementary Figure 4. Total membrane protein for neuronal nitric oxide synthase western blots of human *post-mortem* tissue. Representative total protein in the membrane of western blots used for the quantification of neuronal nitric oxide synthase (nNOS) in the inferior temporal gyrus (ITG) and superior temporal gyrus (STG) (A), and the precuneus (Pre) and primary visual cortex (PVC) (B) from control and late-onset Alzheimer's disease (LOAD) donors and collective sample pool (P). Precision Plus Protein Dual Colour molecular weight marker.


A
Day 0
Day 7
Day 12
Day 19 -
33/39
Day 1
Day 21 +
iPSCs
Embryoid bodies
Neural rosettes
Neurospheres
Neurons
FGF-2
SHH + FGF-8
F5DU
AscA
BDNF + GDNF
cAMP + IGF-1
TeSR-E8
Ni media
Ne media
B
iPSCs
Neural rosettes
Neurospheres
Neurons
Control 1
Control 2
Control 3
LOAD 1
LOAD 2
LOAD 3
fAD

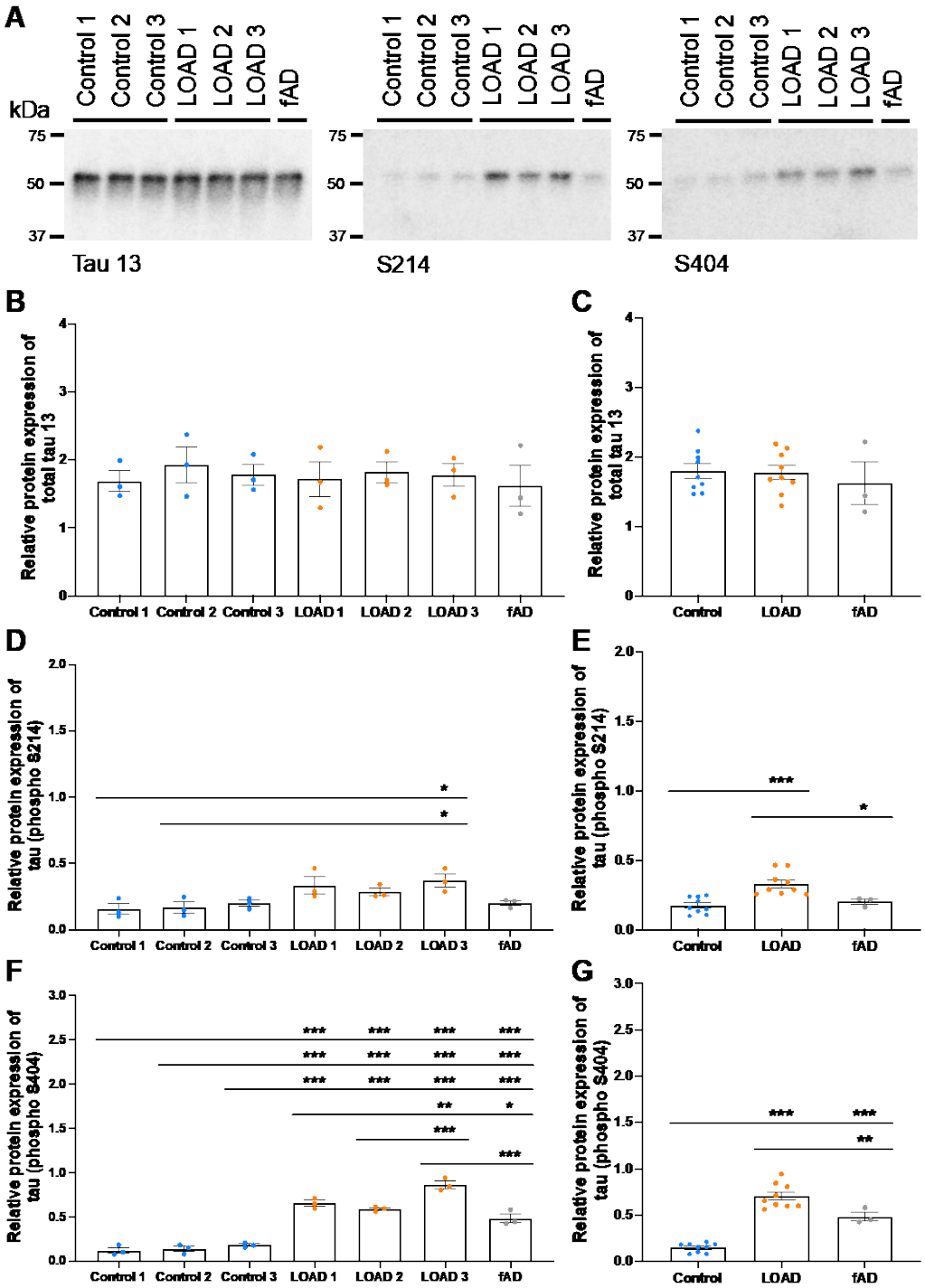
Supplementary Figure 5. Timeline and morphological transition of induced pluripotent stem cells during differentiation to neurons. Schematic diagram (A) detailing key stages of differentiation from induced pluripotent stem cells (iPSCs) to neurons (blue arrow) with corresponding cell culture media (coloured boxes) and growth factors (grey bars) over time. Representative brightfield images (B) from control (Control 1, Control 2, Control 3), late-onset Alzheimer's disease (LOAD 1, LOAD 2, LOAD 3) and familial Alzheimer's disease (fAD) donor lines at key stages of differentiation (iPSCs, neural rosettes, neurospheres, neurons). Images were captured on the last day of each differentiation stage: iPSCs = day 6; neural rosettes = day 18; neurospheres = day 33-39; neurons = day 21 (neuronal differentiation). Scale bars = 200 μm.



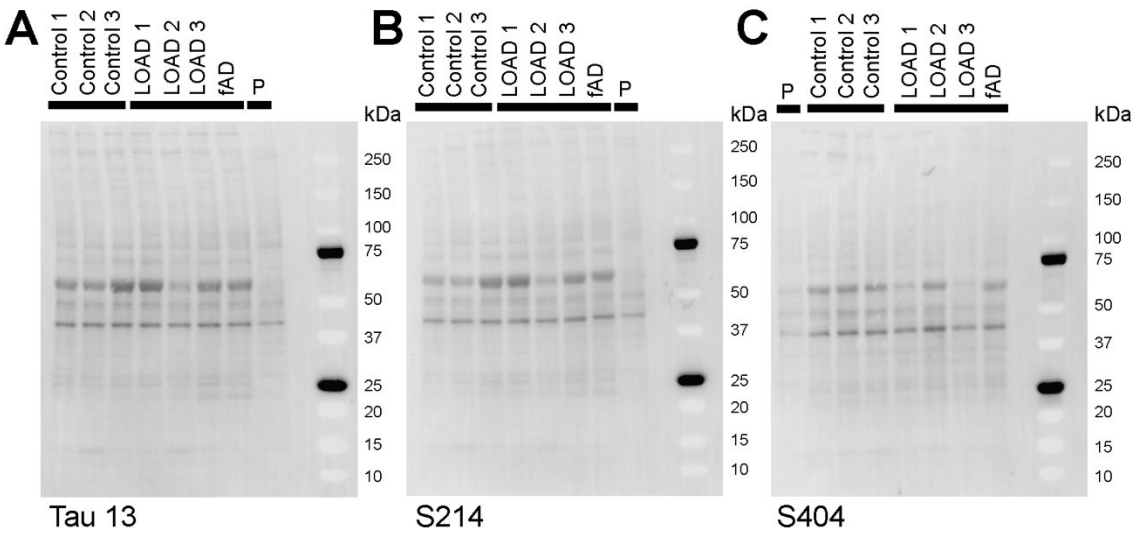
Supplementary Figure 6. Alzheimer's disease neurons have shorter neurites and fewer neurite branch points than control neurons. Mean (± SEM) neurite length (mm/mm^2) (A-B) and neurite branch points (1/mm^2) (C-D) of differentiated neurons in individual donor lines (A, C) and grouped by disease status (B, D) at one (grey), two (blue) and three (orange) weeks of neuronal differentiation. Live-cell images were captured using an IncuCyte Zoom imaging system to track and quantify neurite outgrowth and branch points over time. Data points represent average neurite length or number of neurite branch points from one well of a 24 well plate with 8-16 wells analysed per line per differentiation from three independent differentiations, n = 3. Analysis is Ordinary two-way ANOVA with Tukey's multiple comparisons test. Significant differences are indicated by * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, with blue and orange asterisk representing significant differences between lines at week two and three of differentiation, respectively.



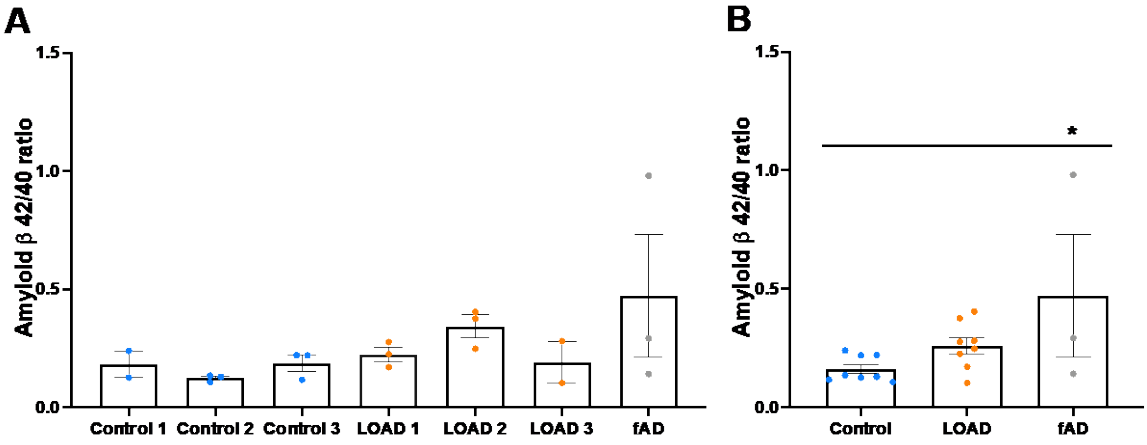
Supplementary Figure 7. IncuCyte NeuroTrack analysis mask for neurite outgrowth and branch points. Representative Incucyte images of differentiated neurons generated from control (A), late-onset Alzheimer's disease (LOAD; B) and familial Alzheimer's disease (fAD; C) donors. Top panel for each donor line is brightfield image and bottom panel is corresponding cell body (yellow) and neurite (purple) mask used for quantification of neurite length and branch points. Scale bars = 300 μm.



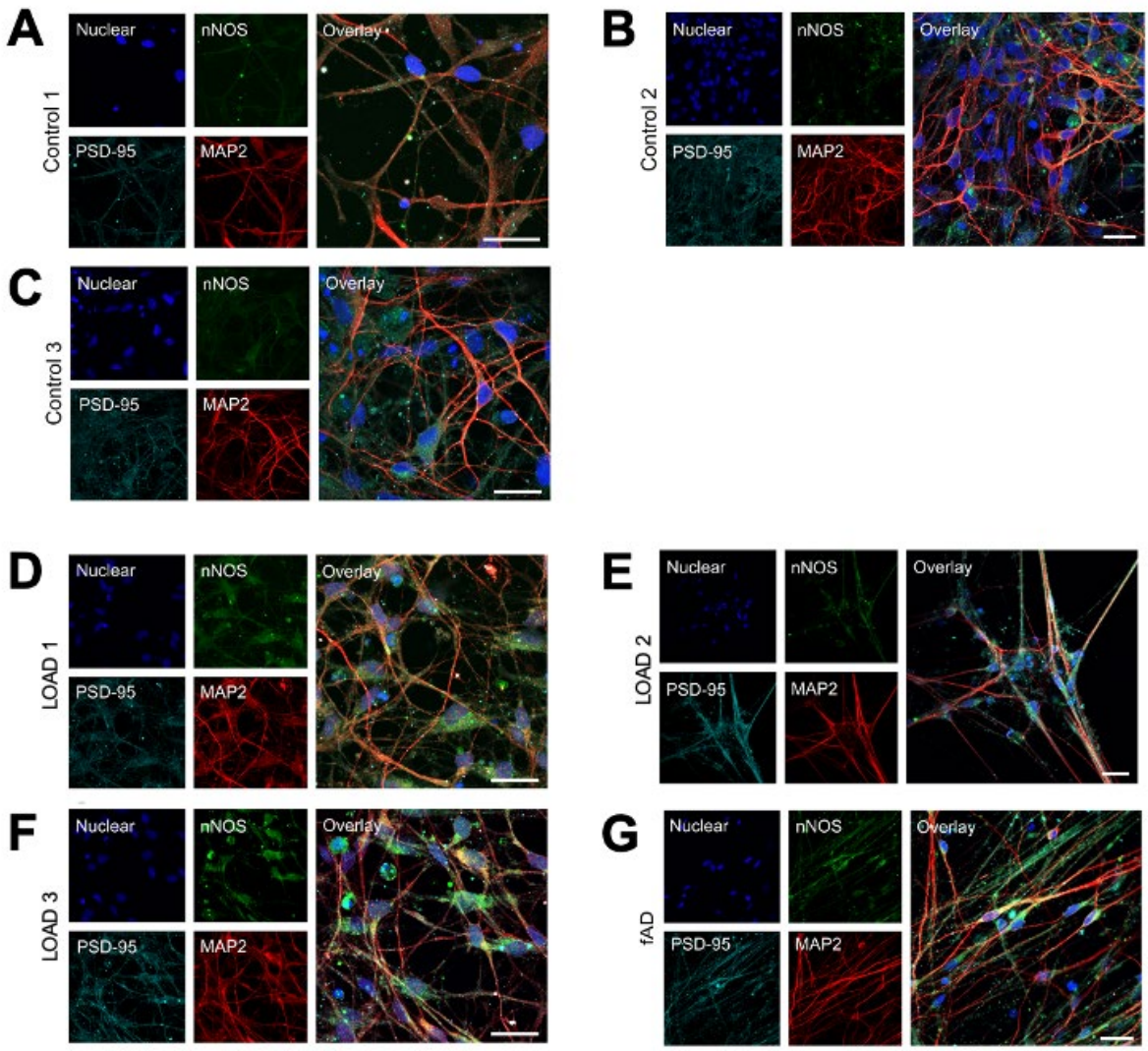
Supplementary Figure 8. Increased relative expression of phosphorylated tau in Alzheimer's disease neurons. Representative western blots (A) and mean (±SEM) relative total tau (tau 13) (B-C), phospho-tau S214 (D-E) and phospho-tau S404 (F-G) protein expression in differentiated neurons from control (blue), late-onset Alzheimer's disease (LOAD; orange) and familial Alzheimer's disease (fAD; grey) in individual donor lines (B, D, F) and grouped by disease status (C, E, G). Neurons were differentiated for three weeks with data points representing mean relative protein expression from one well run in duplicate and normalised to total protein. One well was analysed per line per differentiation from three independent differentiations, n = 3. Analysis is Ordinary one-way ANOVA with Tukey's multiple comparisons test. Significant differences are indicated by * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.



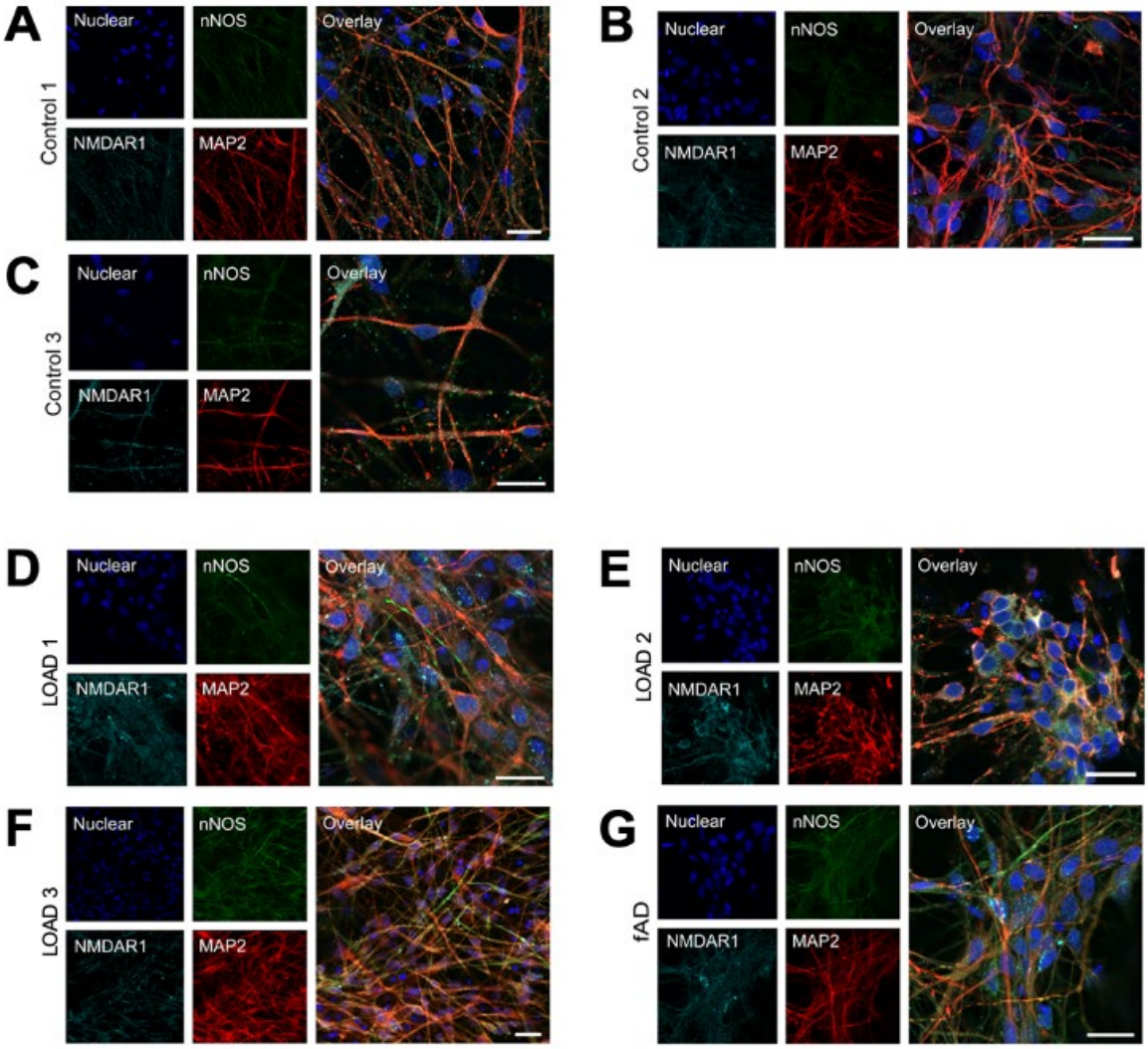
Supplementary Figure 9. Total membrane protein for Tau 13, S214 and S404 western blots of induced pluripotent stem cell derived neurons. Representative total protein in the membrane of western blots used for the quantification of total tau (tau 13) (A), phospho-tau S214 (B) and phosphor-tau S404 (C) in differentiated neurons from control, late-onset Alzheimer's disease (LOAD) and familial Alzheimer's disease (fAD) neurons in individual donor lines and collective sample pool (P). Neurons were differentiated for three weeks. Precision Plus Protein Dual Colour molecular weight marker.



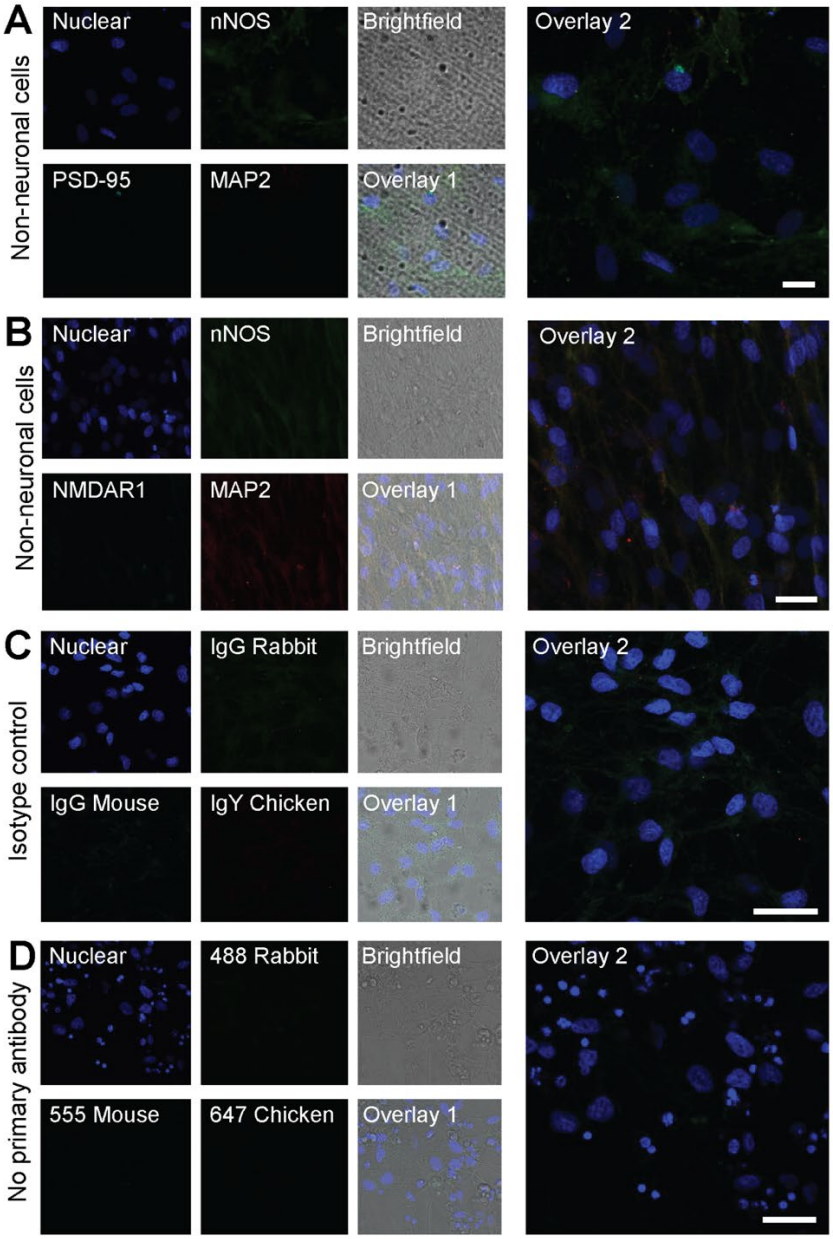
Supplementary Figure 10. Alzheimer's disease neurons show a trend towards a higher amyloid-β 42/40 ratio. Mean (± SEM) of amyloid β 42/40 ratio as measured by ELISA in conditioned media from differentiated neurons from control (blue), late-onset Alzheimer's disease (LOAD; orange) and familial Alzheimer's disease (fAD; grey) individual donor lines (A) and grouped by disease status (B). Neurons were differentiated for four weeks with data points representing mean duplicate readings from one well of a 24 well plate. One well was analysed per line per differentiation from two or three independent differentiations, n = 3. Analysis is Ordinary one-way ANOVA with Tukey's multiple comparisons test. Significant differences are indicated by * $p < 0.05$.



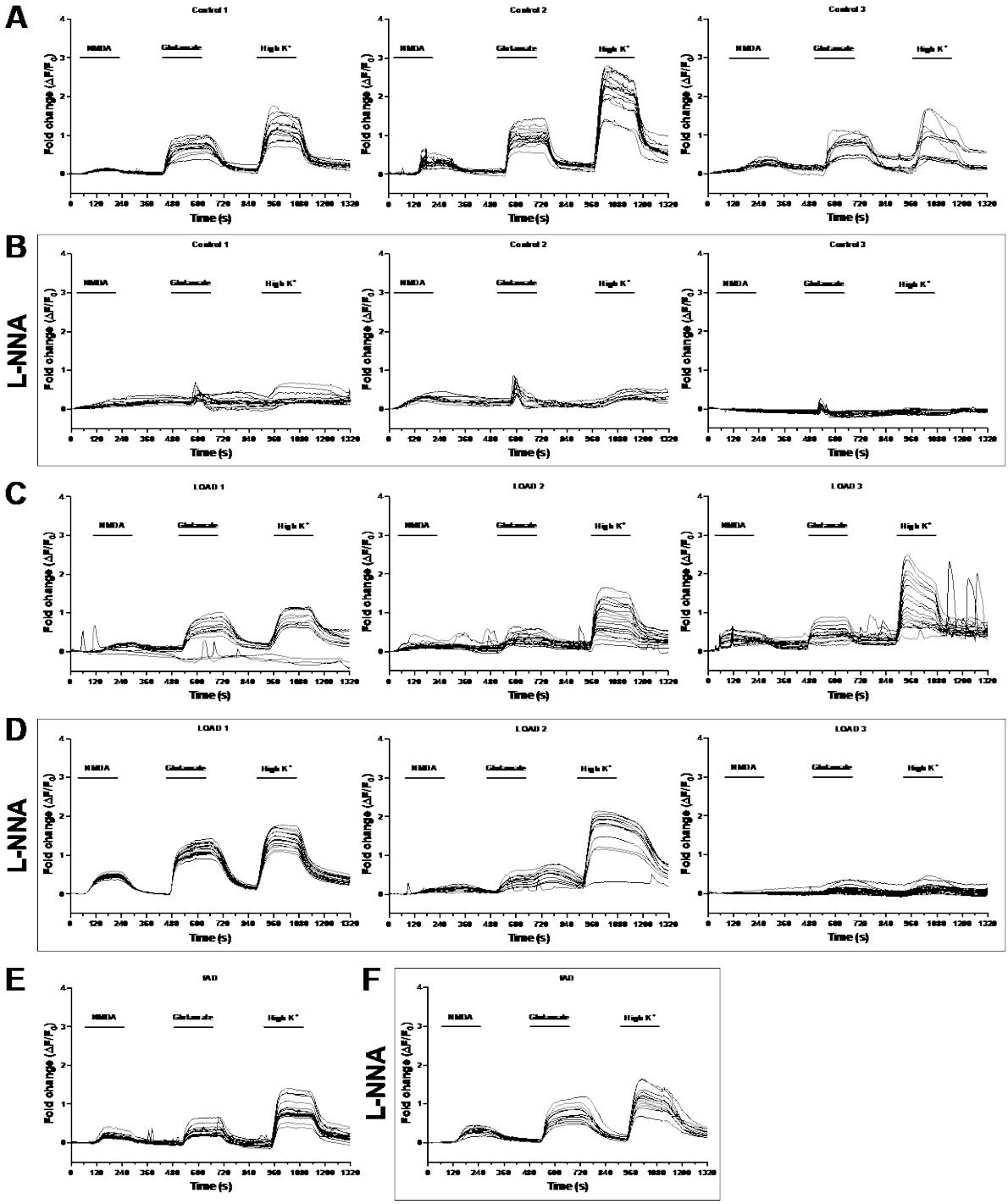
Supplementary Figure 11. Control and Alzheimer's disease neurons express post-synaptic marker PSD-95 and neuronal markers nNOS and MAP2. Representative confocal microscopy images of differentiated neurons generated from control (A-C), late-onset Alzheimer's disease (LOAD; D-F) and familial Alzheimer's disease (fAD; G) donors. All lines were differentiated for a minimum of four weeks and expressed the NO producing enzyme nNOS (green), the post synaptic marker PSD-95 (cyan) and microtubule marker MAP2 (red), with nuclear stain hoechst (blue). Three fields of view were analysed per line per coverslip from two independent differentiations, n = 2. Scale bars = 25 µm.



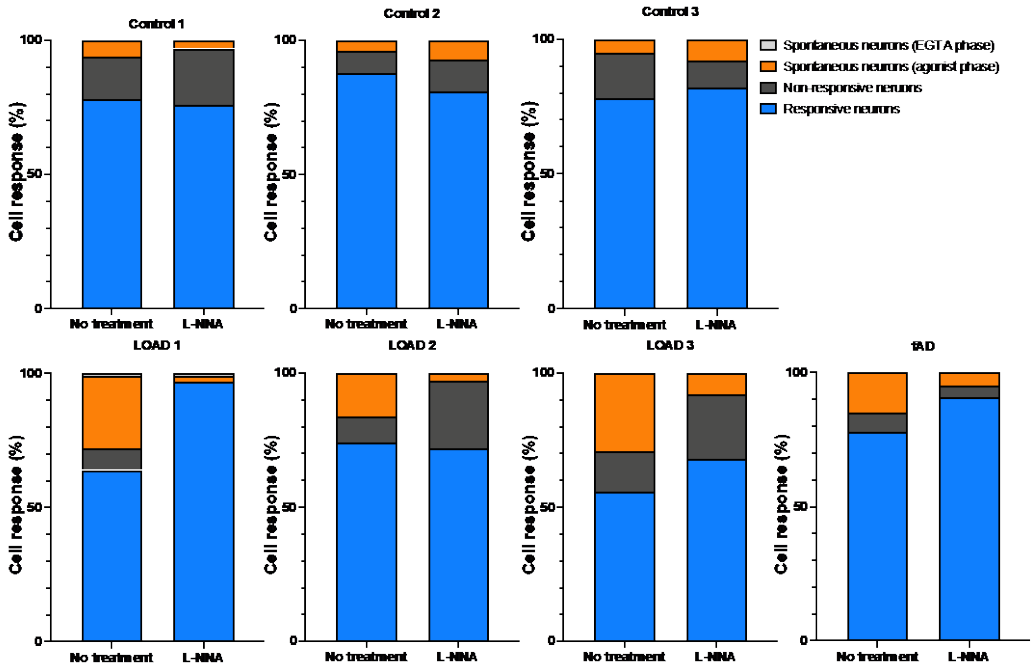
Supplementary Figure 12. Control and Alzheimer's disease neurons express glutamate NMDA receptor and neuronal markers nNOS and MAP2. Representative confocal microscopy images of differentiated neurons generated from control (A-C), late-onset Alzheimer's disease (LOAD; D-F) and familial Alzheimer's disease (fAD; G) donors. All lines were differentiated for a minimum of four weeks and expressed the NO producing enzyme nNOS (green), the glutamate receptor subunit NMDAR1 (cyan) and microtubule marker MAP2 (red), with nuclear stain hoechst (blue). Three fields of view were analysed per line per coverslip from two independent differentiations, n = 2. Scale bars = 25 μm.



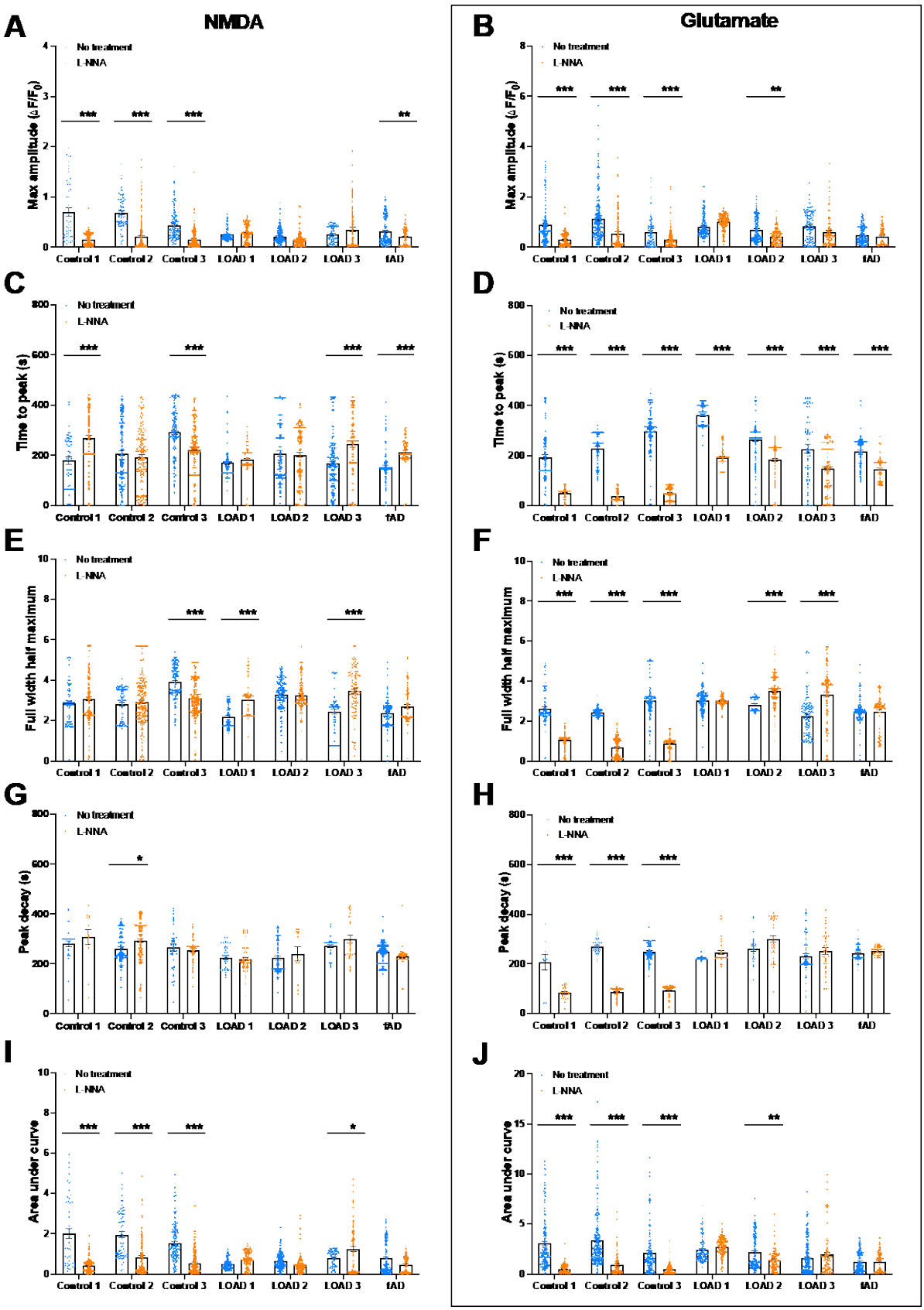
Supplementary Figure 13. Immunocytochemistry controls for induced pluripotent stem cell-derived neuronal cultures. Representative confocal microscopy images of spontaneously differentiated non-neuronal cells (A-B) and differentiated neurons (C-D) used for control immunocytochemical staining. Primary antibodies for post-synaptic density protein (PSD-95) (A), N-methyl-D-aspartate receptor-1 (NMDAR1) (B), in addition to neuronal nitric oxide synthase (nNOS) (A-B) and microtubule associated protein 2 (MAP2) (A-B) were tested for specificity with staining performed on non-neuronal cells. Secondary antibodies were tested for non-specific immunoreactivity and cross-reactivity using Goat anti-Mouse IgG, Goat anti-Rabbit IgG and Goat anti-Chicken IgY isotype antibodies at the same dilution as the respective primary antibody (C), in addition to no primary antibody controls where secondary antibodies Goat anti-Mouse Alexa Fluor 555, Goat anti-Rabbit Alexa Fluor 488 and Goat anti-Chicken Alexa Fluor 647 were run in the absence of primary antibodies (D). All lines were differentiated for a minimum of four weeks and stained with nuclear stain hoechst (blue), with brightfield, brightfield with immunofluorescence (Overlay 1) and immunofluorescence without brightfield (Overlay 2) images also captured. Scale bars = 25 μm.



Supplementary Figure 14. Inhibition of nNOS alters glutamatergic calcium signaling. Representative traces from individual cells of the ratio change in fluorescence intensity over baseline ($\Delta F/F_0$) in response to intracellular calcium fluxes in control (A-B), late-onset Alzheimer's disease (LOAD; C-D) and familial Alzheimer's disease (fAD; E-F) donor lines. In a subset of experiments (B, D, F) cells were pre-treated with nNOS inhibitor L-NNA (50 μM) for 2 h prior to loading with the ratiometric calcium indicator Fura-2 AM. For live cell calcium imaging experiments, cells were perfused with NMDA (10 μM), glutamate (20 μM) and High K^+ (60 mM) (black bars), in addition to EGTA (10 mM) and ionomycin (1.5 μM) (not shown), with a wash in between each agonist. Neurons were differentiated for a minimum of seven weeks.

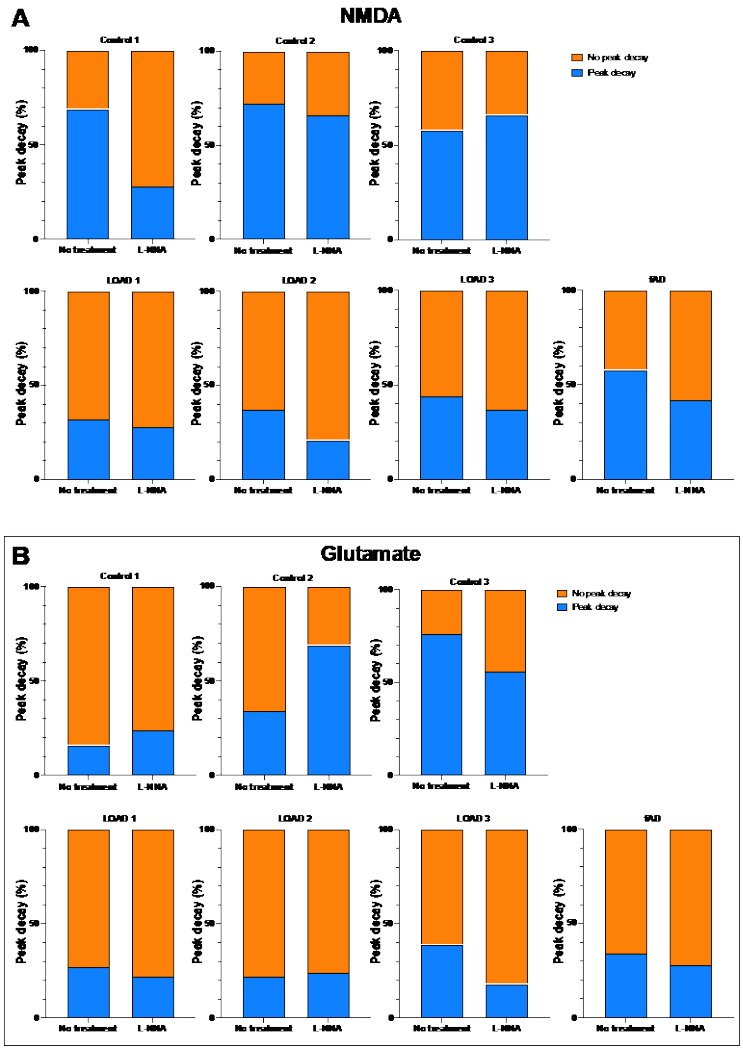


Supplementary Figure 15. Inhibition of nNOS reduced aberrant calcium flux in Alzheimer's disease neurons. Proportion (%) of responsive (blue), non-responsive (dark grey), spontaneously signaling during the agonist phase (orange) and spontaneously signaling during EGTA phase (light grey) neurons from calcium imaging experiments in the absence or presence of L-NNA pre-treatment in individual control, late-onset Alzheimer's disease (LOAD) and familial Alzheimer's disease (fAD) donor lines (A) and grouped by disease status (B). 'Non-responsive' refers to no calcium influx in the presence of an agonist. For live cell calcium imaging experiments, neurons were differentiated for a minimum of seven weeks and a subset of cells were pre-treated with nNOS inhibitor L-NNA (50 µM) for 2 h prior to loading with the ratiometric calcium indicator Fura-2 AM. Cells were perfused with NMDA (10 µM), glutamate (20 µM) and High K^+ (60 mM) (agonist phase), in addition to EGTA (10 mM) and ionomycin (1.5 µM), with a SBS wash in between each agonist. Data represents individual cells with a minimum of 20 cells analysed per coverslip from a minimum of three coverslips per line from three independent differentiations. For the no treatment experiments: Control 1 n = 75; Control 2 n = 81; Control 3 n = 132; LOAD 1 n = 113; LOAD 2 n = 166; LOAD 3 n = 70; fAD n = 152, and for the L-NNA pre-treatment experiments: Control 1 n = 164; Control 2 n = 179; Control 3 n = 219; LOAD 1 n = 151; LOAD 2 n = 149; LOAD 3 n = 110; fAD n = 88.

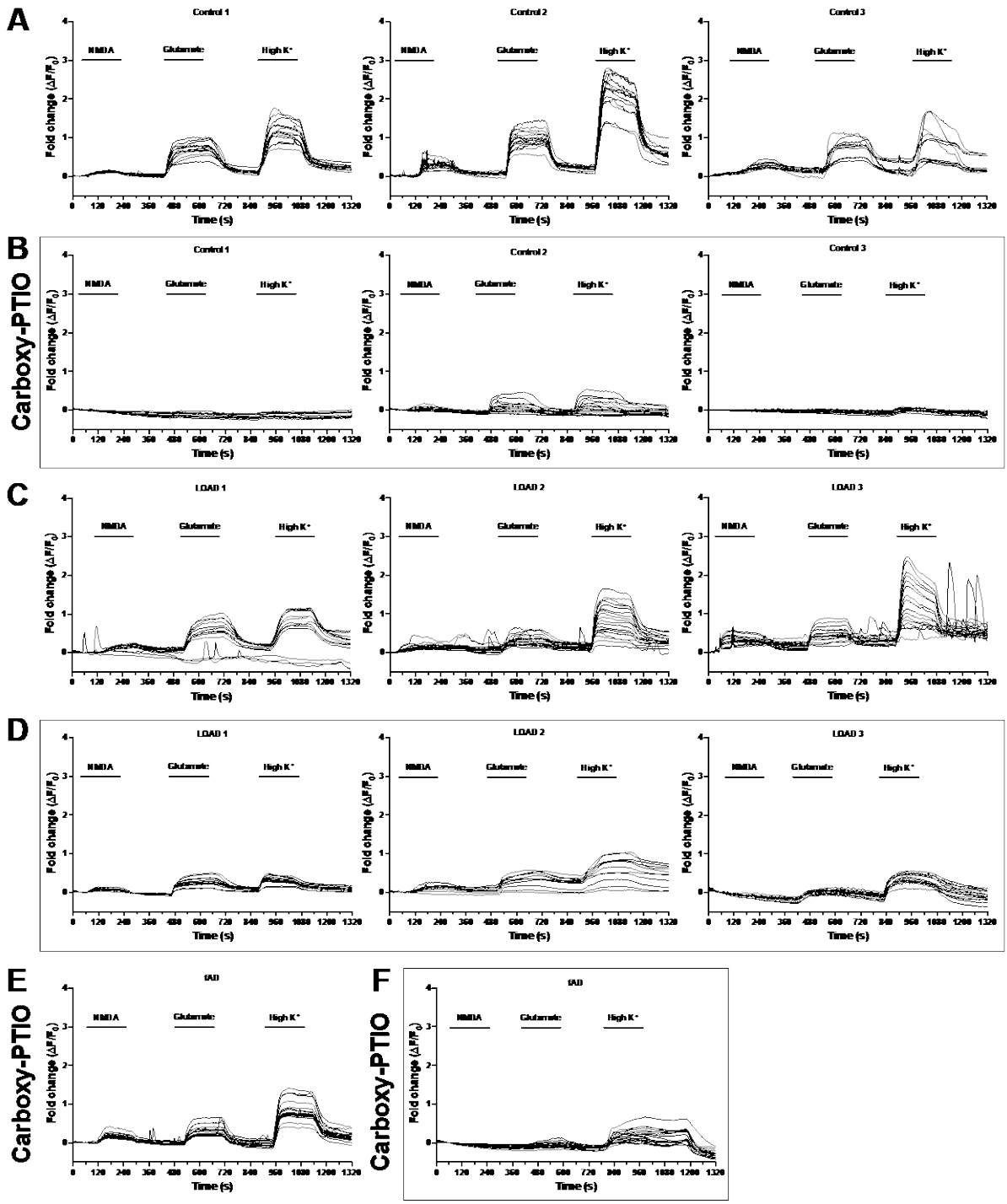


Supplementary Figure 16. Inhibition of neuronal nitric oxide synthase decreased the response to glutamate in control neurons. Mean (± SEM) maximum amplitude of the peak (A-B), the time to peak (C-D), peak duration as full width half maximum of peak (E-F), peak decay (G-H) and global calcium response as the area under the

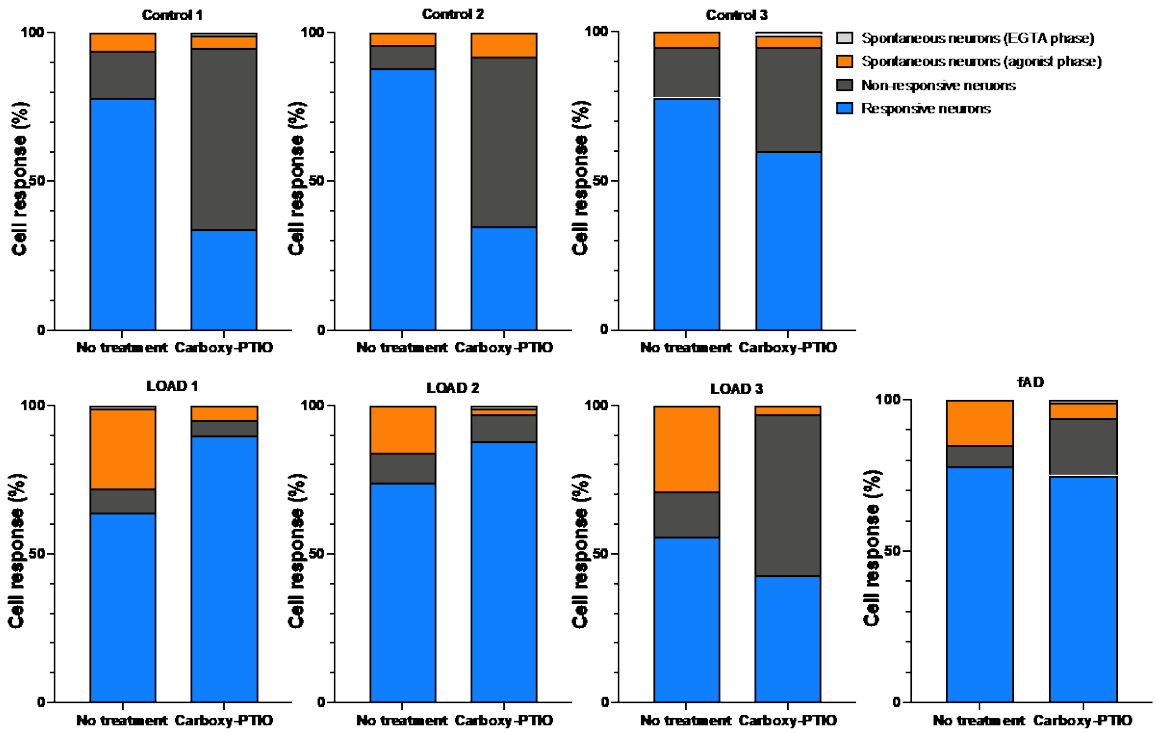
peak curve (I, J) in response to NMDA (A, C, E, G, I) or glutamate (B, D, F, H, J) in the absence (blue) and presence (orange) of L-NNA in differentiated neurons from individual control, late-onset Alzheimer's disease (LOAD) and familial Alzheimer's disease (fAD) donor lines. For live cell calcium imaging experiments, neurons were differentiated for a minimum of seven weeks and a subset of cells were pre-treated with neuronal nitric oxide synthase inhibitor L-NNA (50 μM) for 2 h prior to loading with the ratiometric calcium indicator Fura-2 AM. Cells were perfused with NMDA (10 μM) (shown), glutamate (20 μM), High K^+ (60 mM), EGTA (10 mM) and ionomycin (1.5 μM), with a SBS wash in between each agonist. Data points represent individual cells with a minimum of 20 cells analysed per coverslip from a minimum of three coverslips per line from three independent differentiations. For the no treatment experiments: Control 1 n = 75; Control 2 n = 81; Control 3 n = 132; LOAD 1 = 113; LOAD 2 = 166; LOAD 3 n = 70; fAD n = 152, and for the L-NNA pre-treatment experiments: Control 1 n = 164; Control 2 n = 179; Control 3 n = 219; LOAD 1 = 151; LOAD 2 = 149; LOAD 3 n = 110; fAD n = 88. Analysis is Ordinary two-way ANOVA with Tukey's multiple comparisons test. Significant differences are indicated by * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, with black asterisk representing significant differences between treatment groups for the same line/disease group and blue or orange asterisk representing significant differences between disease groups in the absence or presence of L-NNA pre-treatment, respectively.



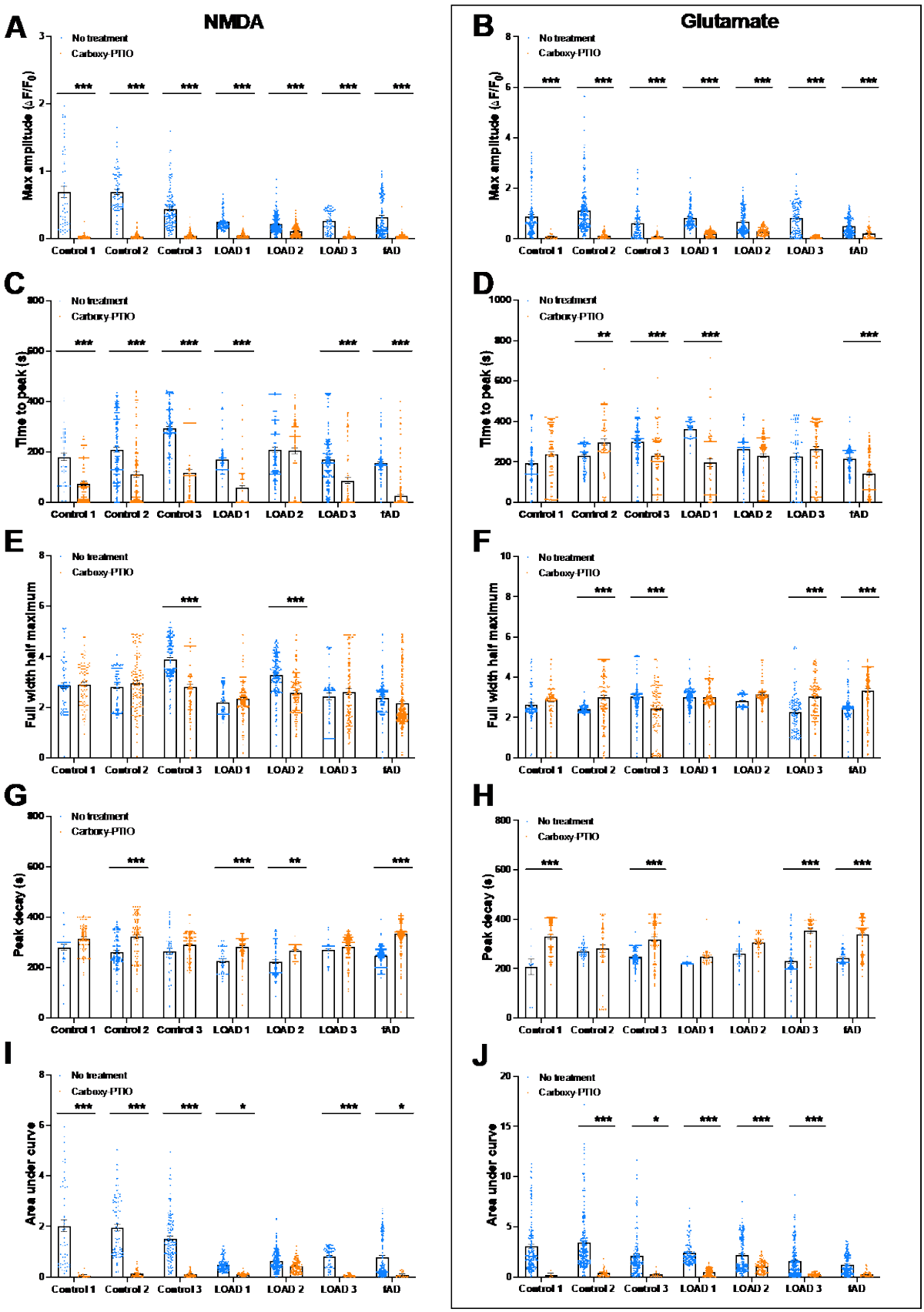
Supplementary Figure 17. Inhibition of nitric oxide synthase had little effect on peak decay in response to NMDA and glutamate in Alzheimer's disease neurons. Proportion (%) of neurons with peak decay in response to NMDA (A) or glutamate (B) in the absence and presence of Carboxy-PTIO in differentiated neurons from individual control, late-onset Alzheimer's disease (LOAD) and familial Alzheimer's disease (fAD) donor lines. For live cell calcium imaging experiments, neurons were differentiated for a minimum of seven weeks and a subset of cells were pre-treated with neuronal nitric oxide synthase inhibitor L-NNA (50 µM) for 2 h prior to loading with the ratiometric calcium indicator Fura-2 AM. Cells were perfused with NMDA (10 µM), glutamate (20 µM) (shown), High K^+ (60 mM), EGTA (10 mM) and ionomycin (1.5 µM), with a SBS wash in between each agonist. Data points represent individual cells with a minimum of 20 cells analysed per coverslip from a minimum of three coverslips per line from three independent differentiations. For the no treatment experiments: Control 1 n = 75; Control 2 n = 81; Control 3 n = 132; LOAD 1 = 113; LOAD 2 = 166; LOAD 3 n = 70; fAD n = 152, and for the L-NNA pre-treatment experiments: Control 1 n = 164; Control 2 n = 179; Control 3 n = 219; LOAD 1 = 151; LOAD 2 = 149; LOAD 3 n = 110; fAD n = 88. Analysis is Ordinary two-way ANOVA with Tukey's multiple comparisons test. Significant differences are indicated by * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, with black asterisk representing significant differences between treatment groups for the same line/disease group and blue or orange asterisk representing significant differences between disease groups in the absence or presence of L-NNA pre-treatment, respectively.



Supplementary Figure 18. Scavenging of NO reduces glutamatergic calcium signaling. Representative traces from individual cells of the ratio change in fluorescence intensity over baseline ($\Delta F/F_0$) in response to intracellular calcium fluxes in control (A-B), late-onset Alzheimer's disease (LOAD; C-D) and familial Alzheimer's disease (fAD; E-F) donor lines. In a subset of experiments (B, D, F) cells were pre-treated with NO scavenger carboxy-PTIO (30 µM) for 2 h prior to loading with the ratiometric calcium indicator Fura-2 AM. For live cell calcium imaging experiments, cells were perfused with NMDA (10 µM), glutamate (20 µM) and High K^+ (60 mM) (black bars), in addition to EGTA (10 mM) and ionomycin (1.5 µM) (not shown), with a SBS wash in between each agonist. Neurons were differentiated for a minimum of seven weeks.

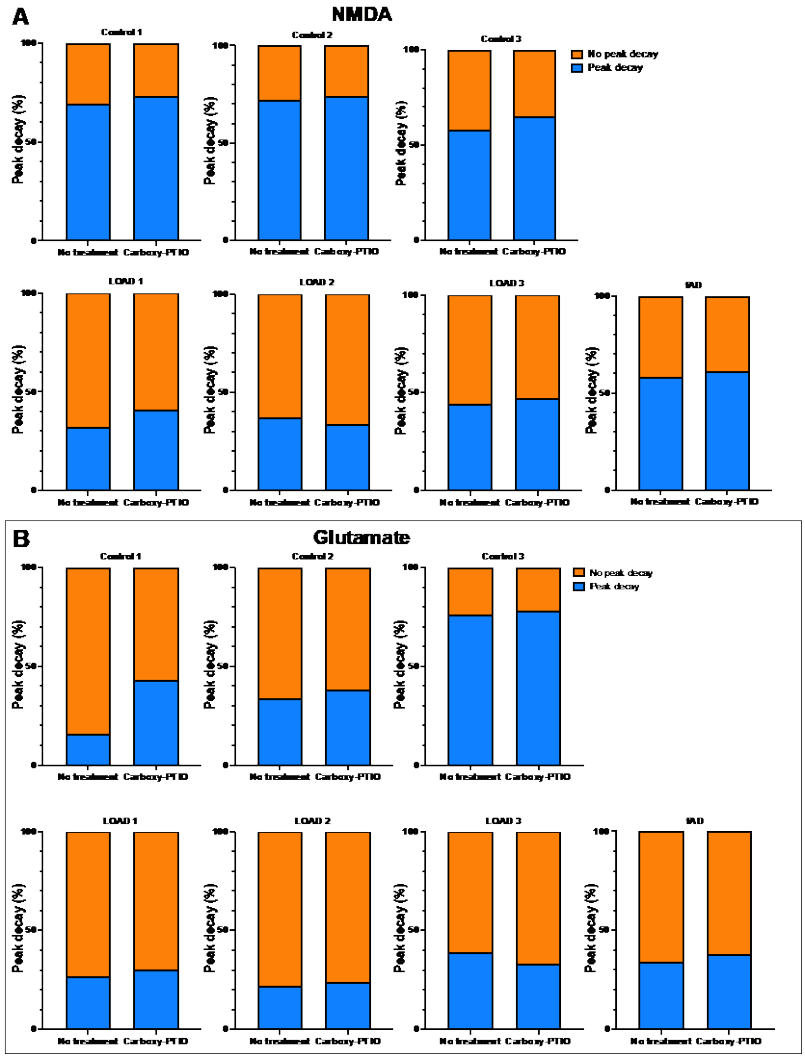


Supplementary Figure 19. Scavenging of NO reduced aberrant calcium flux in Alzheimer's disease neurons and increased the proportion of non-responsive neurons. Proportion (%) of responsive (blue), non-responsive (dark grey), spontaneously signaling during the agonist phase (orange) and spontaneously signaling during EGTA phase (light grey) neurons from calcium imaging experiments in the absence or presence of Carboxy-PTIO pre-treatment in individual control, late-onset Alzheimer's disease (LOAD) and familial Alzheimer's disease (fAD) donor lines (A) and grouped by disease status (B). 'Non-responsive' refers to no calcium flux in the presence of an agonist. For live cell calcium imaging experiments, neurons were differentiated for a minimum of seven weeks and a subset of cells were pre-treated with NO scavenger Carboxy-PTIO (30 µM) for 2 h prior to loading with the ratiometric calcium indicator Fura-2 AM. Cells were perfused with NMDA (10 µM), glutamate (20 µM) and High K^+ (60 mM) (agonist phase), in addition to EGTA (10 mM) and ionomycin (1.5 µM), with a SBS wash in between each agonist. Data represents individual cells with a minimum of 20 cells analysed per coverslip from a minimum of three coverslips per line from three independent differentiations. For the no treatment experiments: Control 1 n = 75; Control 2 n = 81; Control 3 n = 132; LOAD 1 n = 113; LOAD 2 n = 166; LOAD 3 n = 70; fAD n = 152, and for the Carboxy-PTIO pre-treatment experiments: Control 1 n = 91; Control 2 n = 108; Control 3 n = 111; LOAD 1 n = 164; LOAD 2 n = 142; LOAD 3 n = 129; fAD n = 286.

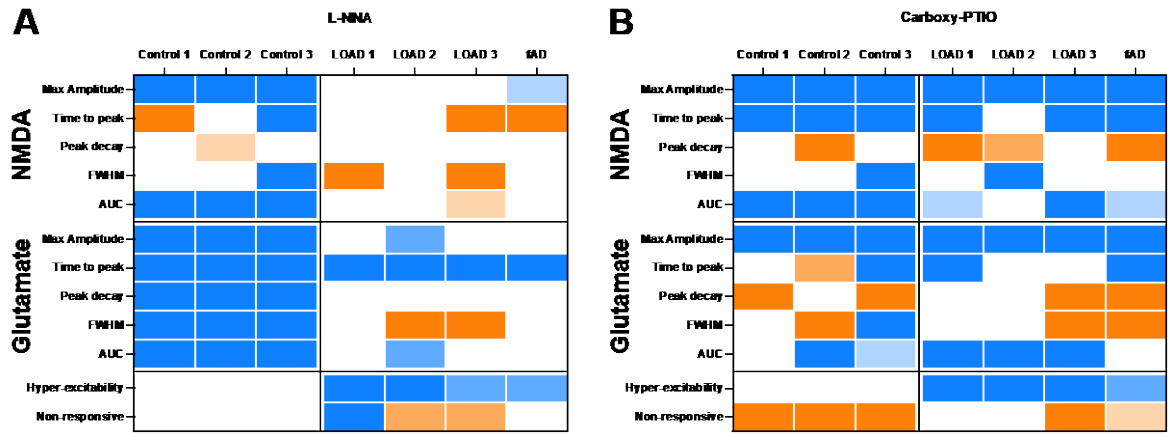


Supplementary Figure 20. Scavenging of nitric oxide reduced the time to peak and increased the peak decay in response to NMDA in control and Alzheimer's disease neurons. Mean (± SEM) maximum amplitude of the peak (A-B), the time to peak (C-D), peak duration as full width half maximum of peak (E-F), peak decay (G-H)

and global calcium response as the area under the peak curve (I, J) in response to NMDA (A, C, E, G, I) or glutamate (B, D, F, H, J) in the absence (blue) and presence (orange) of Carboxy-PTIO in differentiated neurons from individual control, late-onset Alzheimer's disease (LOAD) and familial Alzheimer's disease (fAD) donor lines. For live cell calcium imaging experiments, neurons were differentiated for a minimum of seven weeks and a subset of cells were pre-treated with nitric oxide scavenger Carboxy-PTIO (30 μM) for 2 h prior to loading with the ratiometric calcium indicator Fura-2 AM. Cells were perfused with NMDA (10 μM) (shown), glutamate (20 μM), High K^+ (60 mM), EGTA (10 mM) and ionomycin (1.5 μM), with a SBS wash in between each agonist. Data points represent individual cells with a minimum of 20 cells analysed per coverslip from a minimum of three coverslips per line from three independent differentiations. For the no treatment experiments: Control 1 n = 75; Control 2 n = 81; Control 3 n = 132; LOAD 1 = 113; LOAD 2 = 166; LOAD 3 n = 70; fAD n = 152, and for the Carboxy-PTIO pre-treatment experiments: Control 1 n = 91; Control 2 n = 108; Control 3 n = 111; LOAD 1 = 164; LOAD 2 = 142; LOAD 3 n = 129; fAD n = 286. Analysis is Ordinary two-way ANOVA with Tukey's multiple comparisons test. Significant differences are indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, with black asterisk representing significant differences between treatment groups for the same line/disease group and blue or orange asterisk representing significant differences between disease groups in the absence or presence of Carboxy-PTIO pre-treatment, respectively.



Supplementary Figure 21. Scavenging of nitric oxide had little effect on peak decay in response to NMDA and glutamate in neurons. Proportion (%) of neurons with peak decay in response to NMDA (A) or glutamate (B) in the absence and presence of Carboxy-PTIO in differentiated neurons from individual control, late-onset Alzheimer's disease (LOAD) and familial Alzheimer's disease (fAD) donor lines. For live cell calcium imaging experiments, neurons were differentiated for a minimum of seven weeks and a subset of cells were pre-treated with nitric oxide scavenger Carboxy-PTIO (30 µM) for 2 h prior to loading with the ratiometric calcium indicator Fura-2 AM. Cells were perfused with NMDA (10 µM), glutamate (20 µM) (shown), High K^+ (60 mM), EGTA (10 mM) and ionomycin (1.5 µM), with a SBS wash in between each agonist. Data points represent individual cells with a minimum of 20 cells analysed per coverslip from a minimum of three coverslips per line from three independent differentiations. For the no treatment experiments: Control 1 n = 75; Control 2 n = 81; Control 3 n = 132; LOAD 1 = 113; LOAD 2 = 166; LOAD 3 n = 70; fAD n = 152, and for the Carboxy-PTIO pre-treatment experiments: Control 1 n = 91; Control 2 n = 108; Control 3 n = 111; LOAD 1 = 164; LOAD 2 = 142; LOAD 3 n = 129; fAD n = 286. Analysis is Ordinary two-way ANOVA with Tukey's multiple comparisons test. Significant differences are indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, with black asterisk representing significant differences between treatment groups for the same line/disease group and blue or orange asterisk representing significant differences between disease groups in the absence or presence of Carboxy-PTIO pre-treatment, respectively.



Supplementary Figure 22. Changes to glutamatergic calcium responses following inhibition of nNOS or scavenging of NO in control and Alzheimer's disease neurons. Summary heatmap of neuronal calcium responses to NMDA and glutamate post treatment with nNOS inhibitor L-NNA (A) or Carboxy-PTIO (B) in individual control, late-onset Alzheimer's disease (LOAD) or familial AD (fAD) donor lines. Calcium response parameters quantified are the maximum amplitude ($\Delta F/F_0$), time to peak (s), time of peak decay (s), full width half maximum (FWHM) as a proxy for response duration and the area under the curve (AUC) as a proxy for the global calcium response. Tiles represent a significant increase (orange), decrease (blue) or no difference (white) in the calcium response to NMDA and glutamate following pre-treatment with either L-NNA or carboxy-PTIO as compared to untreated neurons. The degree of significant difference on the colour map is indicated by * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.