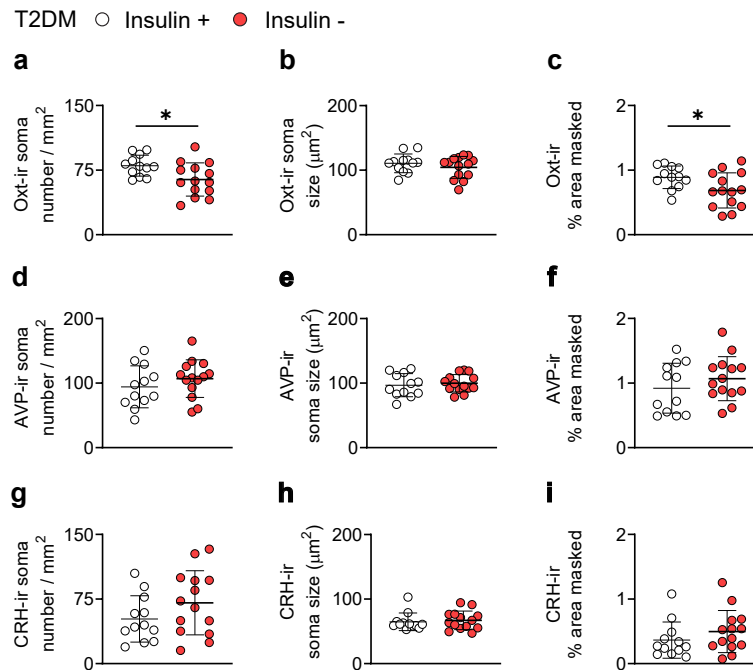


# Reduction of oxytocin-containing neurons and enhanced glymphatic activity in the hypothalamic paraventricular nucleus of patients with type 2 diabetes mellitus

Felipe Correa-da-Silva<sup>1,2</sup>, Martin J. Kalsbeek<sup>1,2</sup>, Femke S. Gadella<sup>1</sup>, Jorn Oppersma<sup>1</sup>, Wei Jiang<sup>1,2</sup>, Samantha E.C. Wolff<sup>1</sup>, Nikita L. Korpel<sup>3</sup>, Dick F. Swaab<sup>2</sup>, Eric Fliers<sup>1</sup>, Andries Kalsbeek<sup>1,2,3</sup>, Chun-Xia Yi<sup>1,2,3</sup>

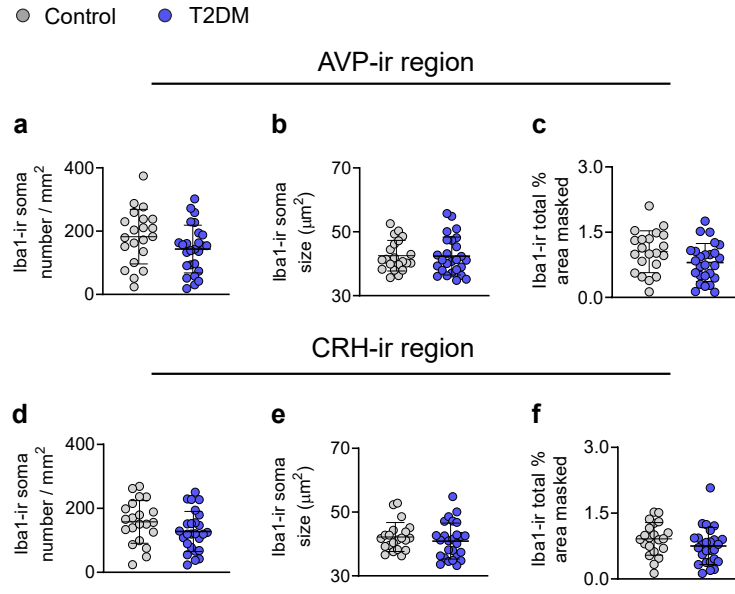
## Electronic Supplementary Materials



**Supplementary Fig. 1**

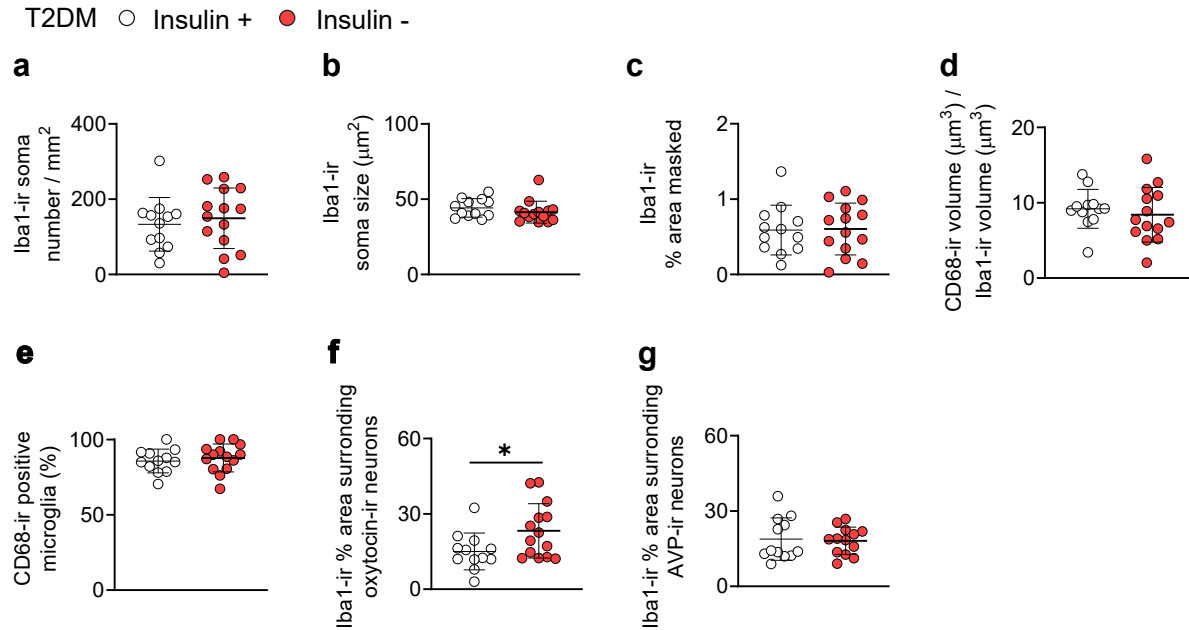
**The antidiabetic treatment-associated effect on PVN neuronal populations. Comparison between T2DM individuals with or without insulin treatment.** (a) Oxt-ir soma number / mm<sup>2</sup> (neuronal density), (b) Oxt-ir average soma size and (c) relative masked area by positive Oxt-ir cells. (d) Quantitative analysis of (f) AVP-ir soma number / mm<sup>2</sup> (neuronal density), (e) AVP-ir average soma size and (f) relative masked area by positive AVP-ir cells. (g) CRH-ir soma number / mm<sup>2</sup> (neuronal density), (h) CRH-ir average soma size and (i) relative masked area by positive CRH-ir particles. Note that Oxt-ir neurons density and relative masked area are reduced in untreated T2DM individuals. AVP = arginine-vasopressin; CRH= corticotrophin releasing hormone; ir = immunoreactivity; PVN = paraventricular nucleus; T2DM = type 2 diabetic mellitus. Data are represented as mean ± SEM. \* p< 0.05. Significance was calculated using Student's t test in a-i. T2DM insulin + : n = 12, T2DM insulin - : n=14.





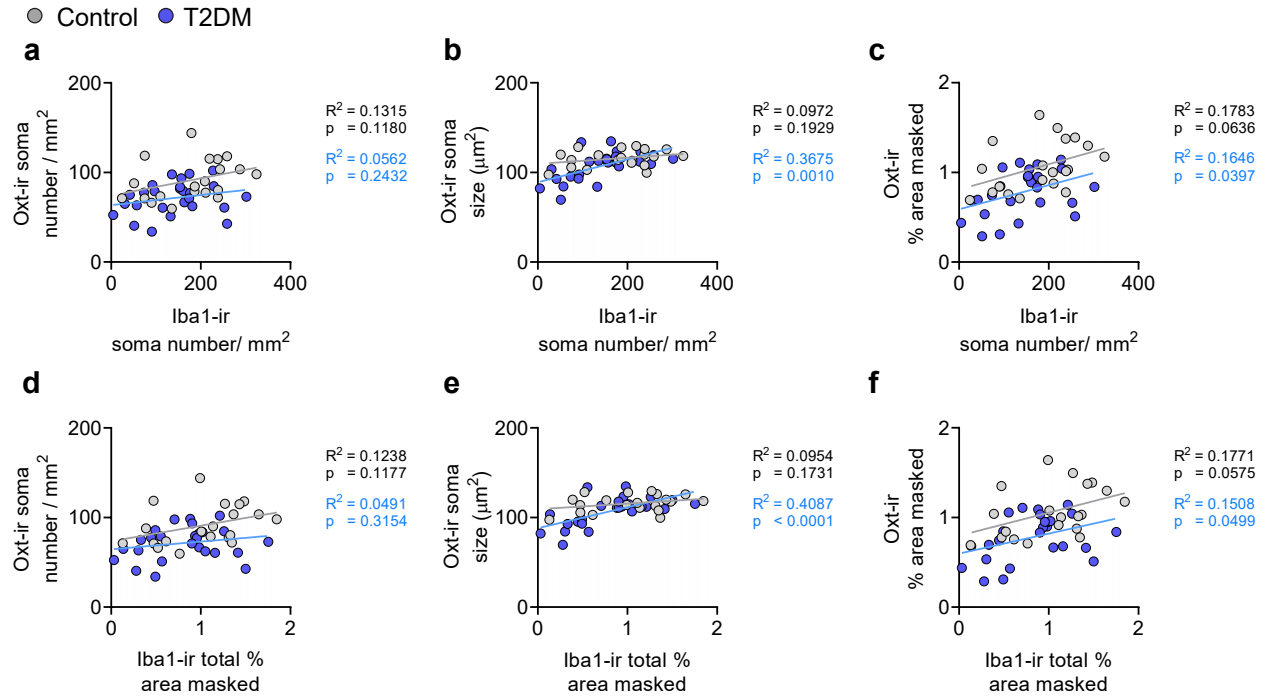
**Supplementary Fig. 3**

**Microglia number is unchanged in the PVN of T2DM individuals. Quantitative analysis of Iba1-ir in the PVN (AVP-ir region) of control and T2DM subjects.** Plot of microglial parameters (a) Iba1-ir soma number / mm<sup>2</sup> (cell density), (b) Iba1-ir average soma size and (c) relative masked area by positive microglial cells. Quantitative analysis of Iba1-ir in the PVN (CRH-ir region) of control and T2DM subjects. Plot of microglial parameters (d) Iba1-ir soma number / mm<sup>2</sup> (cell density), (e) Iba1-ir average soma size and (f) relative masked area of positive microglial cells. AVP = arginine-vasopressin; CRH= corticotrophin releasing hormone; ir = immunoreactivity; PVN = paraventricular nucleus; Iba1 = ionized calcium-binding adapter molecule 1; T2DM = type 2 diabetic mellitus. Data are represented as mean ± SEM. Control: n = 20, T2DM: n = 26.



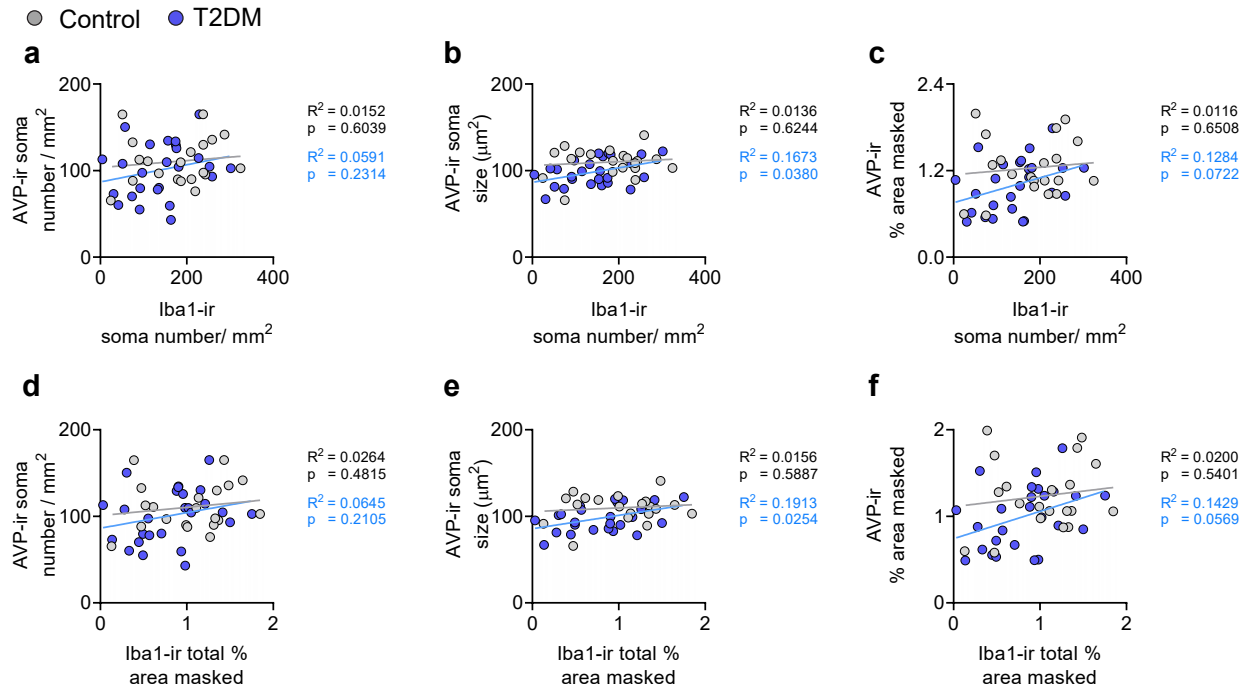
**Supplementary Fig. 4**

**The antidiabetic treatment-associated effect on PVN microglia. Comparison between T2DM individuals with or without insulin treatment.** (a) Iba1-ir soma number / mm<sup>2</sup> (cell density), (b) Iba1-ir average soma size and (c) relative masked area by positive microglial cells. (d) Volume percentage of CD68-ir in relation to Iba1-ir and (e) Quantitative analysis of CD68-ir positive microglia in percentage. Mean relative area of masked (%) of Iba1-ir particles surrounding Oxt-ir neurons (f) and AVP-ir neurons (g) within 10 µm. Please note that microglial features are unaltered in the PVN irrespective of insulin treatment, except for Iba1-ir particles abundance around Oxt neurons. These data suggest closer microglial contact in T2DM untreated subjects. Oxt = oxytocin; AVP = arginine-vasopressin; ir = immunoreactivity; PVN = paraventricular nucleus; Iba1 = ionized calcium-binding adapter molecule 1; CD68 = cluster of differentiation 68; T2DM = type 2 diabetic mellitus. Data are represented as mean ± SEM. \* p< 0.05. Significance was calculated using Student's t test in a-g, T2DM insulin + : n = 12, T2DM insulin - : n=14.



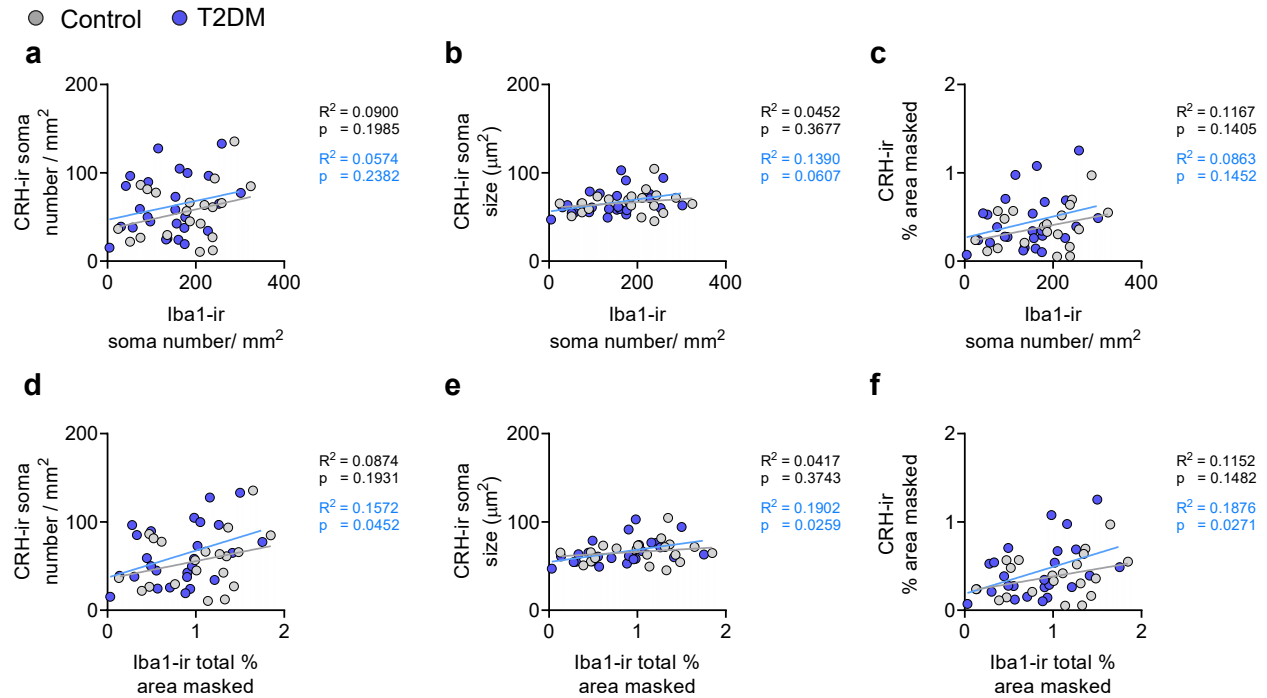
**Supplementary Fig. 5**

**Analysis of oxytocin-ir (Oxt-ir) neurons in relation to microglial (Iba1-ir) cells in the PVN of control and T2DM subjects.** Plots of (a) Oxt-ir neurons density in relation to Iba1-ir microglial density; (b) Oxt-ir neurons soma size in relation to Iba1-ir microglial density and (c) relative masked area by Oxt-ir cells in relation to Iba1-ir microglial density. Plots of (d) Oxt-ir neurons density in relation to relative masked area of Iba1-ir microglial cells; (e) Oxt-ir neurons soma size in relation to relative masked area of Iba1-ir microglial cells (f) Oxt-ir relative masked area in relation to relative masked area of Iba1-ir microglial cells Oxt = oxytocin; Iba1 = ionized calcium-binding adapter molecule 1; PVN = paraventricular nucleus; ir = immunoreactivity; CSF = cerebrospinal fluid; post-abs=post absorptive; T2DM = type 2 diabetic mellitus. Control: n = 20, T2DM: n = 26.



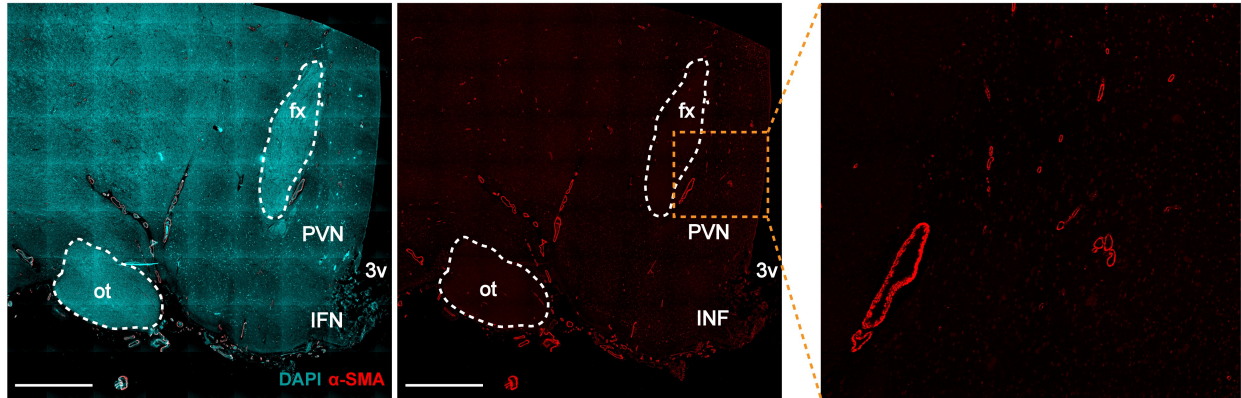
**Supplementary Fig. 6**

**Analysis of AVP-ir (AVP-ir) neurons in relation to microglial (Iba1-ir) cells in the PVN of the hypothalamus of control and T2DM subjects.** Plots of (a) AVP-ir neurons density in relation to Iba1-ir microglial density; (b) AVP-ir neurons soma size in relation to Iba1-ir microglial density and (c) relative masked area by AVP-ir cells in relation to Iba1-ir microglial density. Plots of (d) AVP-ir neurons density in relation to relative masked area of Iba1-ir microglial cells; (e) AVP-ir neurons soma size in relation to relative masked area of Iba1-ir microglial cells (f) AVP-ir relative masked area in relation to relative masked area of Iba1-ir microglial cells. AVP= arginine vasopressin; Iba1 = ionized calcium-binding adapter molecule 1; ir = immunoreactivity; PVN = paraventricular nucleus; CSF = cerebrospinal fluid; post-abs=post absorptive; T2DM = type 2 diabetic mellitus. Control: n = 20, T2DM: n = 26.



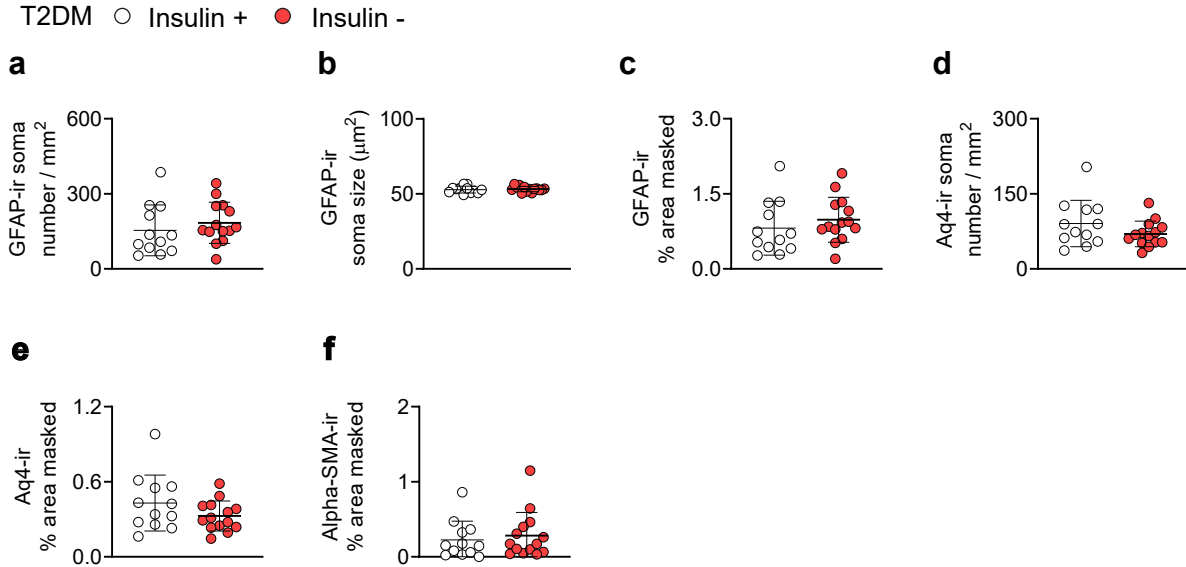
**Supplementary Fig. 7**

**Analysis of corticotropin-ir (CRH-ir) neurons in relation to microglial (Iba1-ir) cells in the PVN of the hypothalamus of control and T2DM subjects.** Plots of (a) CRH-ir neurons density in relation to Iba1-ir microglial density; (b) CRH-ir neurons soma size in relation to Iba1-ir microglial density and (c) relative masked area by CRH-ir cells in relation to Iba1-ir microglial density. Plots of (d) CRH-ir neurons density in relation to relative masked area of Iba1-ir microglial cells; (e) CRH-ir neurons soma size in relation to relative masked area of Iba1-ir microglial cells (f) CRH-ir relative masked area in relation to relative masked area of Iba1-ir microglial cells. CRH = corticotropin releasing hormone; Iba1 = ionized calcium-binding adapter molecule 1; PVN = paraventricular nucleus; ir = immunoreactivity; CSF = cerebrospinal fluid; post-abs=post absorptive; T2DM = type 2 diabetic mellitus. Control: n = 20, T2DM: n = 26.



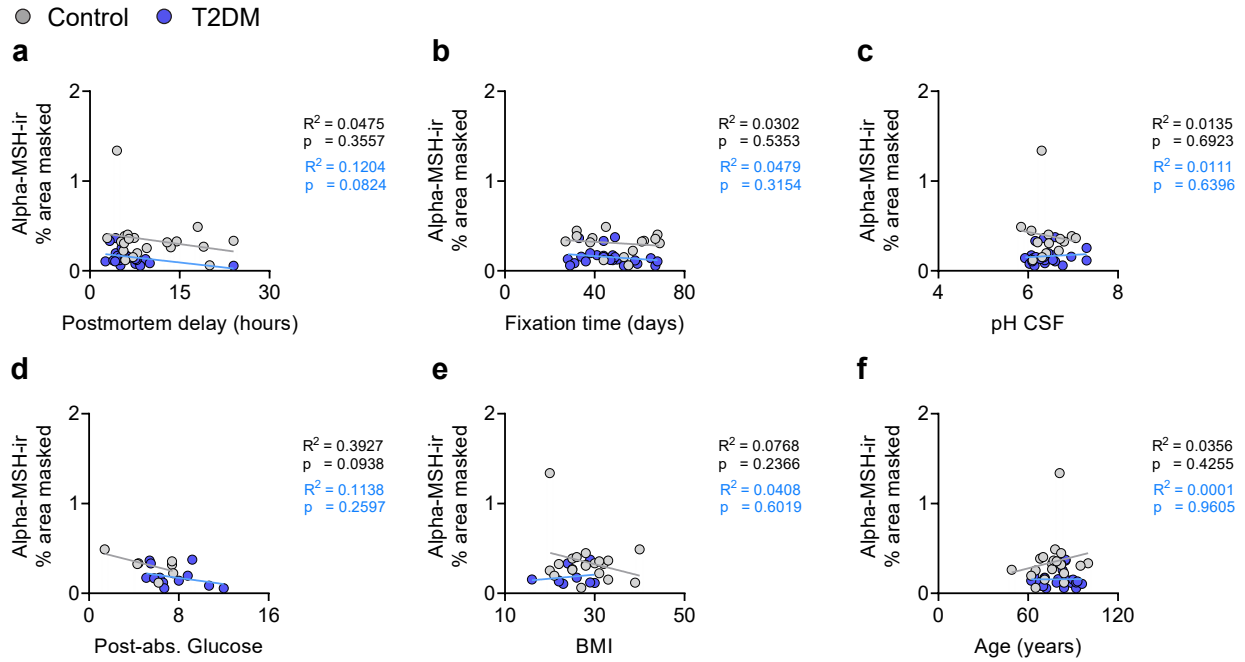
**Supplementary Fig. 8**

**Representative image of alpha-smooth muscle actin-ir (alpha-SMA) in the human hypothalamus.** Alpha-SMA and DAPI co-labelling (left image), single alpha-SMA channel (center). Yellow dashed-line partially frames the PVN, with provided higher magnification of alpha-SMA-ir pattern. 3V = third ventricle; fx =fornix; INF = infundibular nucleus; PVN = paraventricular nucleus; ot = optic tract. Scale bar = 2 mm.



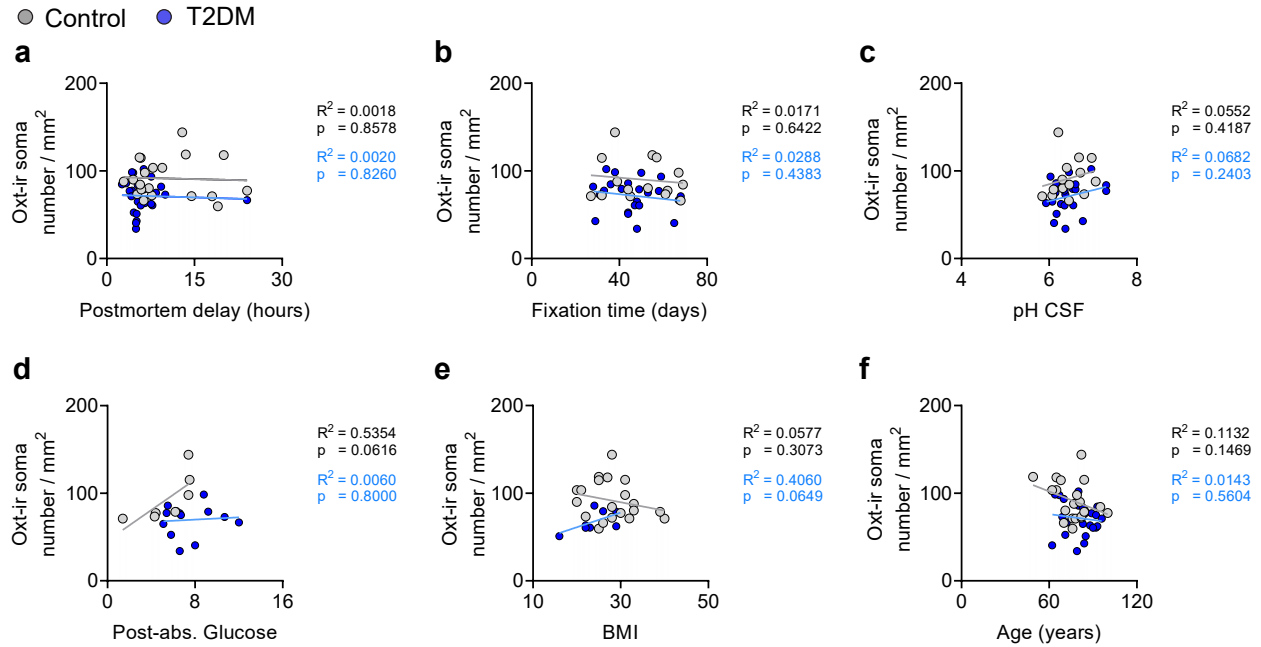
**Supplementary Fig. 9**

**The antidiabetic treatment-associated effect on PVN astroglia and glymphatic system. Comparison between T2DM individuals with or without insulin treatment.** (a) GFAP-ir soma number / mm<sup>2</sup> (cell density), (b) GFAP-ir average soma size and (c) relative masked area by positive astrocytic particles. (d) Quantitative analysis of Aq4-ir cells / mm<sup>2</sup> (density) and (e) relative masked area by Aq4-ir. (f) Quantitative analysis of alpha-SMA-ir relative masked area by positive endothelial cells. Please note that astrocytic and glymphatic system components are not affected by insulin treatment in T2DM subjects. GFAP = glial fibrillary acid protein; Aq4 = aquaporin 4; alpha-SMA = alpha smooth muscle actin; ir = immunoreactivity; PVN = paraventricular nucleus; T2DM = type 2 diabetic mellitus. Data are represented as mean ± SEM. T2DM insulin + : n = 12, T2DM insulin - : n=14.



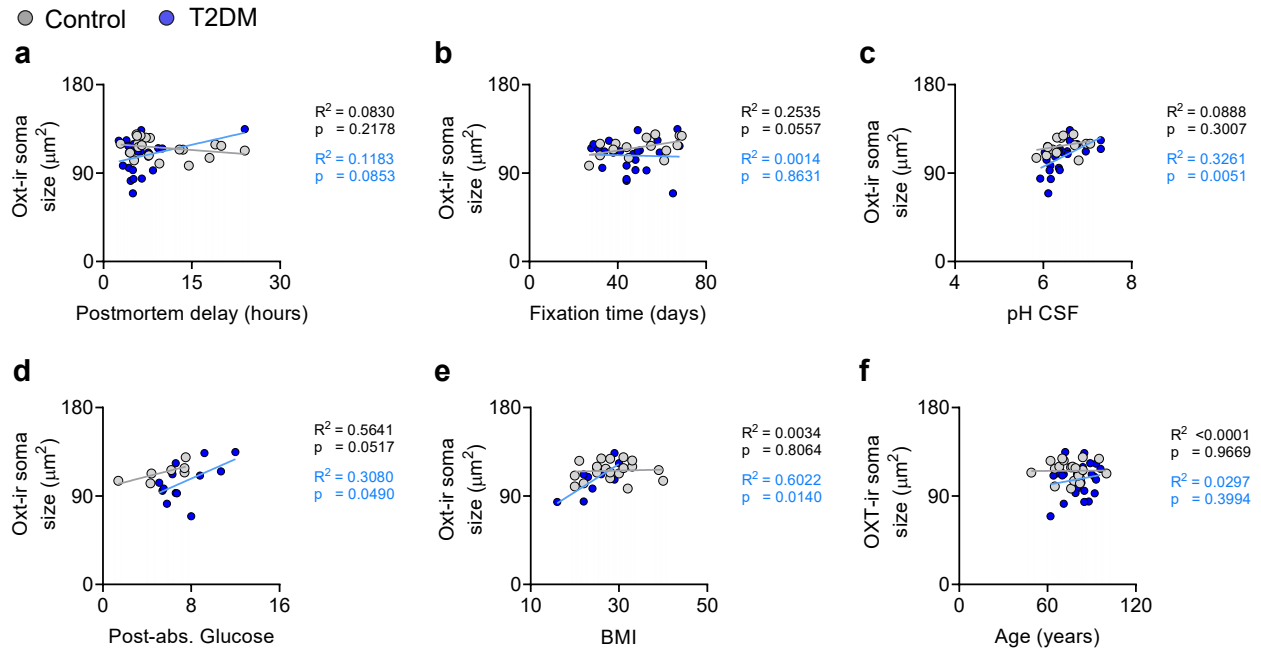
**Supplementary Fig. 10**

**Co-founder analysis of alpha-MSH-ir fibers area masked in the PVN of control and T2DM subjects.** (a-f) Plots of alpha-MSH-ir relative masked area in the PVN according to (a) postmortem delay, (b) fixation time, (c) pH of CSF, (d) post absorptive glucose, (e) and body mass index (BMI) and (f) age. Alpha-MSH = alpha-melanocortin stimulating hormone; PVN = paraventricular nucleus; ir = immunoreactivity; CSF = cerebrospinal fluid; post-abs=post absorptive; T2DM = type 2 diabetic mellitus. Control: n = 20, T2DM: n = 26.



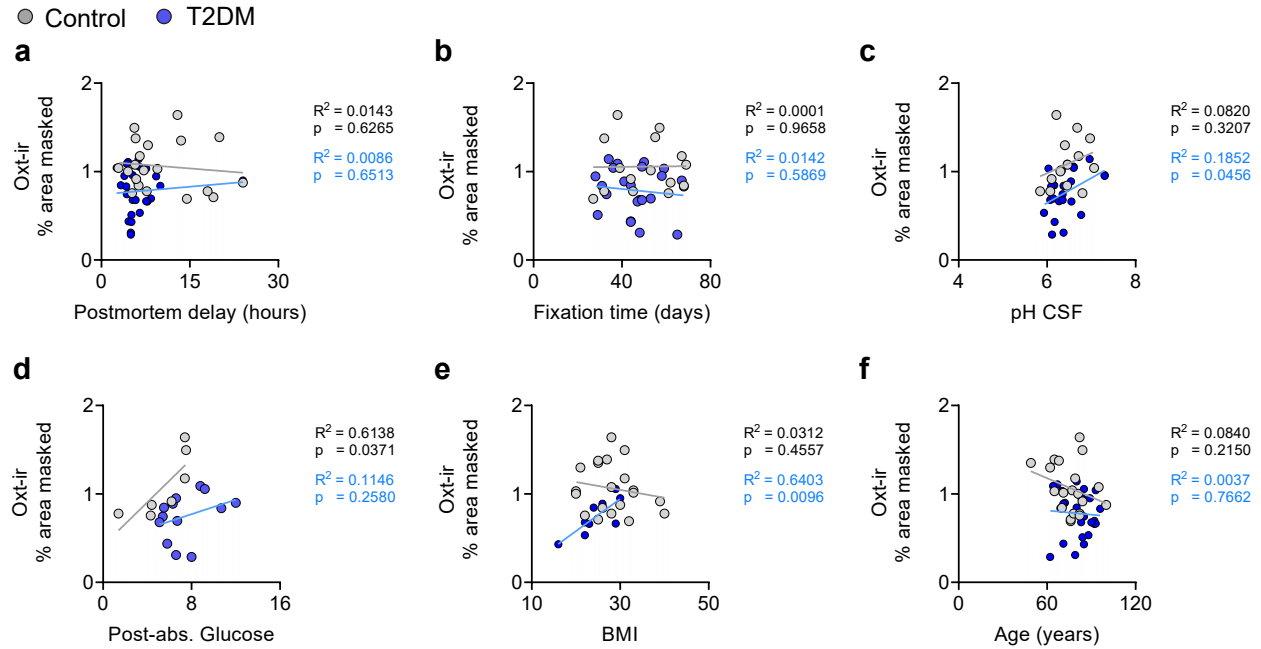
**Supplementary Fig. 11**

**Co-founder analysis of oxytocin-ir (Oxt-ir) neurons density in the PVN of control and T2DM subjects.** (a-f) Plots of Oxt-ir soma number/mm<sup>2</sup> in the PVN according to (a) postmortem delay, (b) fixation time, (c) pH of CSF, (d) post absorptive glucose, (e) and body mass index (BMI) and (f) age. Oxt = oxytocin; PVN = paraventricular nucleus; ir = immunoreactivity; CSF = cerebrospinal fluid; post-abs=post absorptive; T2DM = type 2 diabetic mellitus. Control: n = 20, T2DM: n = 26.



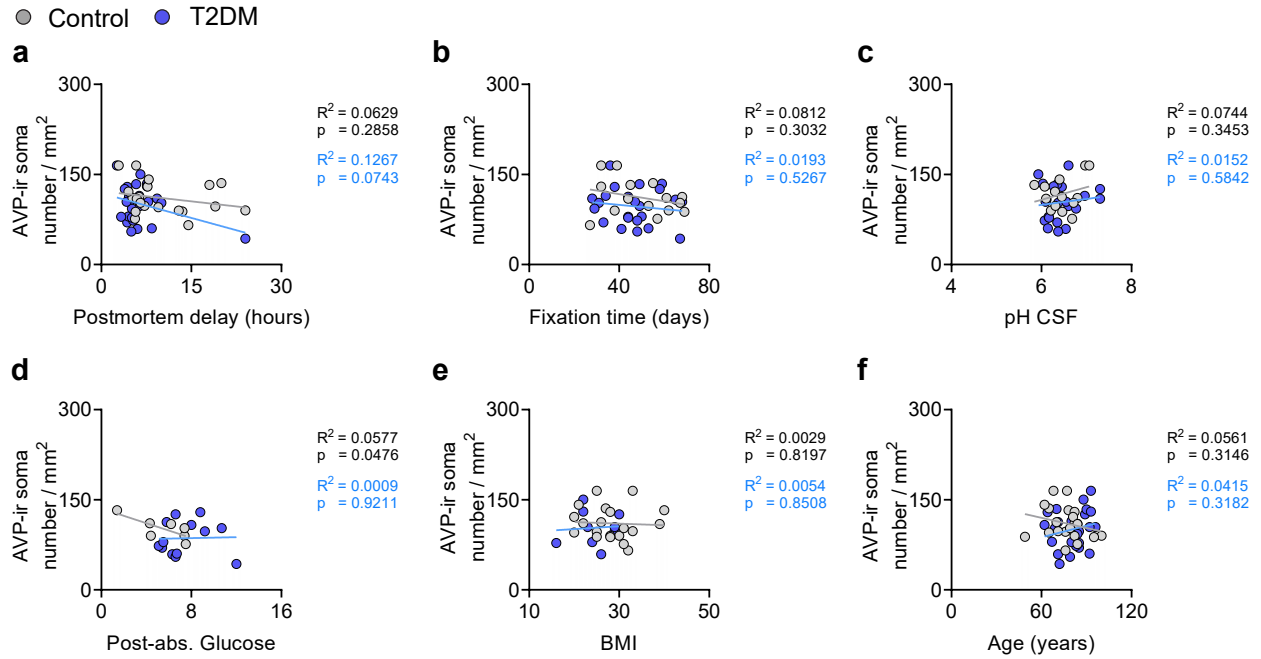
**Supplementary Fig. 12**

**Co-founder analysis of oxytocin-ir (Oxt-ir) soma size in the PVN of control and T2DM subjects.** (a-f) Plots of oxytocin-ir soma size in the PVN according to (a) postmortem delay, (b) fixation time, (c) pH of CSF, (d) post absorptive glucose, (e) and body mass index (BMI) and (f) age. Oxt = oxytocin; PVN = paraventricular nucleus; ir = immunoreactivity; CSF = cerebrospinal fluid; post-abs=post absorptive; T2DM = type 2 diabetic mellitus. Control: n = 20, T2DM: n = 26.



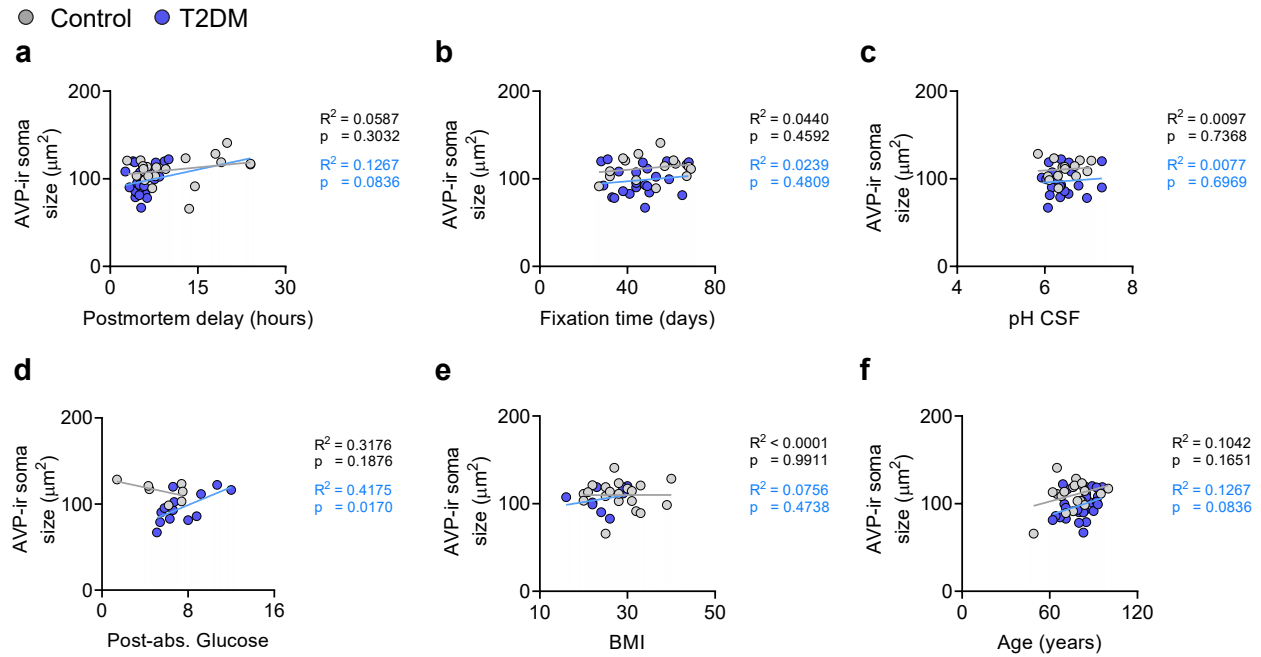
**Supplementary Fig. 13**

**Co-founder analysis of total area masked by positive oxytocin-ir (Oxt-ir) cells in the PVN of control and T2DM subjects. (a-f)** Plots of xt-ir relative masked area in the PVN according to (a) postmortem delay, (b) fixation time, (c) pH of CSF, (d) post absorptive glucose, (e) and body mass index (BMI) and (f) age. Oxt = oxytocin; ir = immunoreactivity; CSF = cerebrospinal fluid; post-abs=post absorptive; T2DM = type 2 diabetic mellitus. Control: n = 20, T2DM: n = 26.



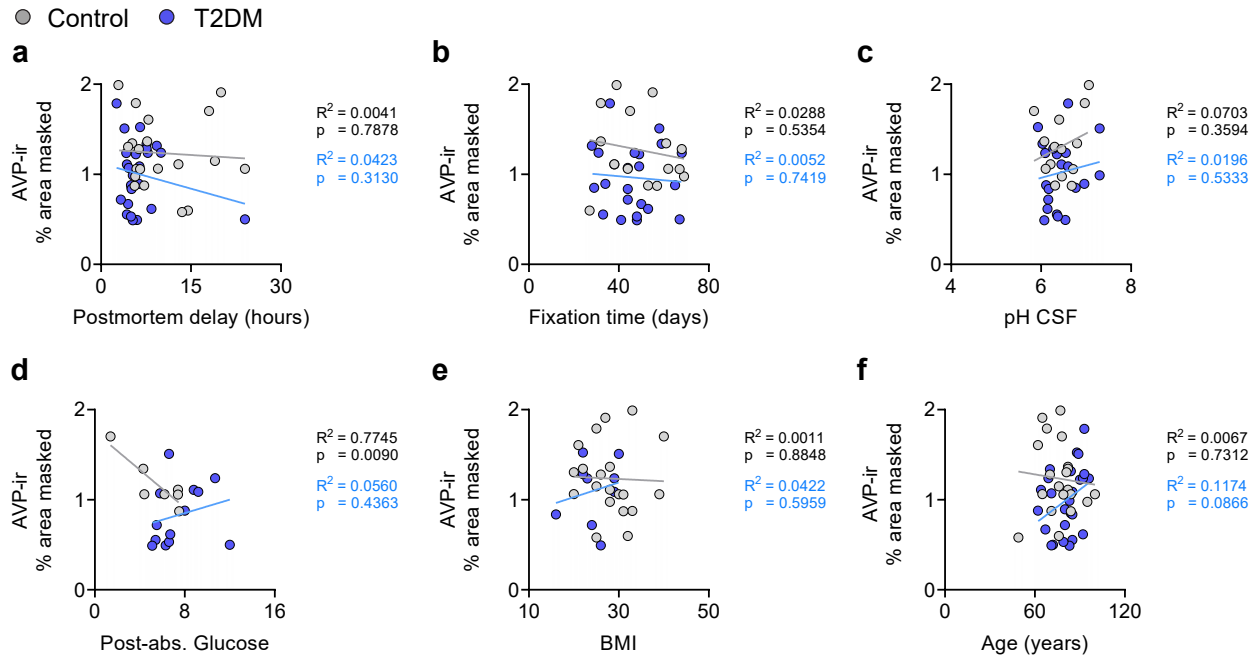
**Supplementary Fig. 14**

**Co-founder analysis of arginine-vasopressin-ir (AVP-ir) neurons density in the PVN of control and T2DM subjects.** (a-f) Plots of AVP-ir soma number/mm<sup>2</sup> in the PVN according to (a) postmortem delay, (b) fixation time, (c) pH of CSF, (d) post absorptive glucose, (e) and body mass index (BMI) and (f) age. AVP = arginine-vasopressin; PVN = paraventricular nucleus; ir = immunoreactivity; CSF = cerebrospinal fluid; post-abs=post absorptive; T2DM = type 2 diabetic mellitus. Control: n = 20, T2DM: n = 26.



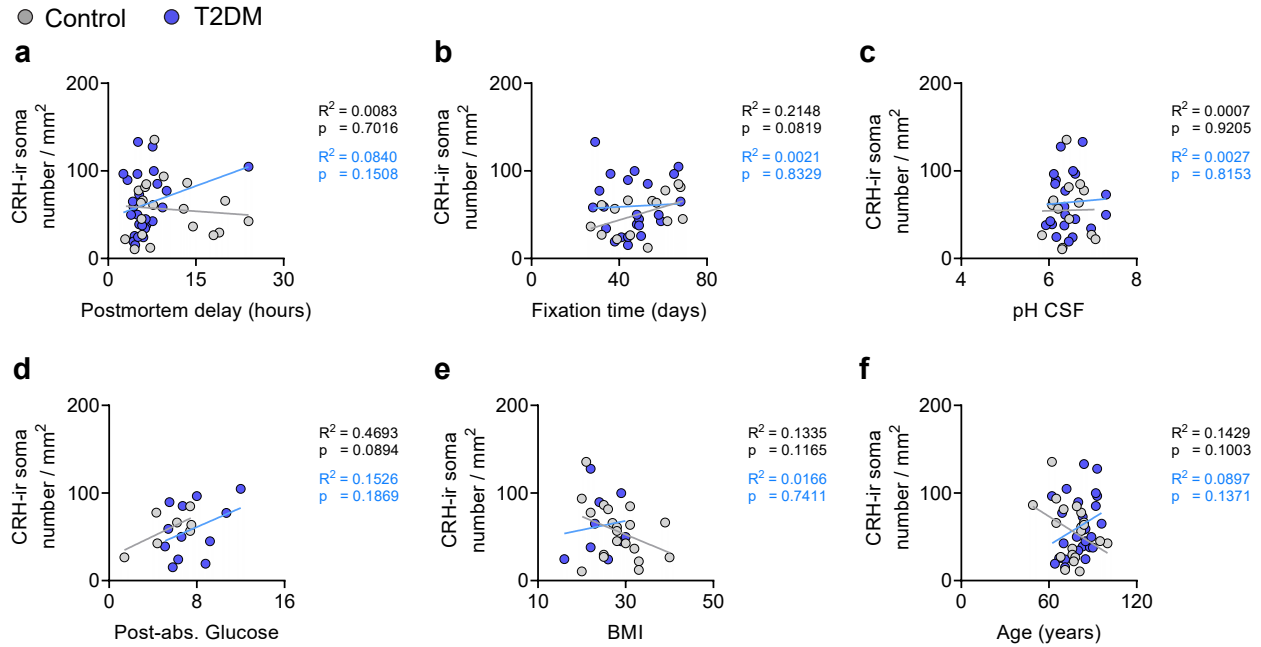
**Supplementary Fig. 15**

**Co-founder analysis of arginine-vasopressin-ir (AVP-ir) soma size in the PVN of control and T2DM subjects.** (a-f) Plots of AVP-ir soma size in the PVN according to (a) postmortem delay, (b) fixation time, (c) pH of CSF, (d) post absorptive glucose, (e) and body mass index (BMI) and (f) age. AVP = arginine-vasopressin; PVN = paraventricular nucleus; ir = immunoreactivity; CSF = cerebrospinal fluid; post-abs=post absorptive; T2DM = type 2 diabetic mellitus. Control: n = 20, T2DM: n = 26.



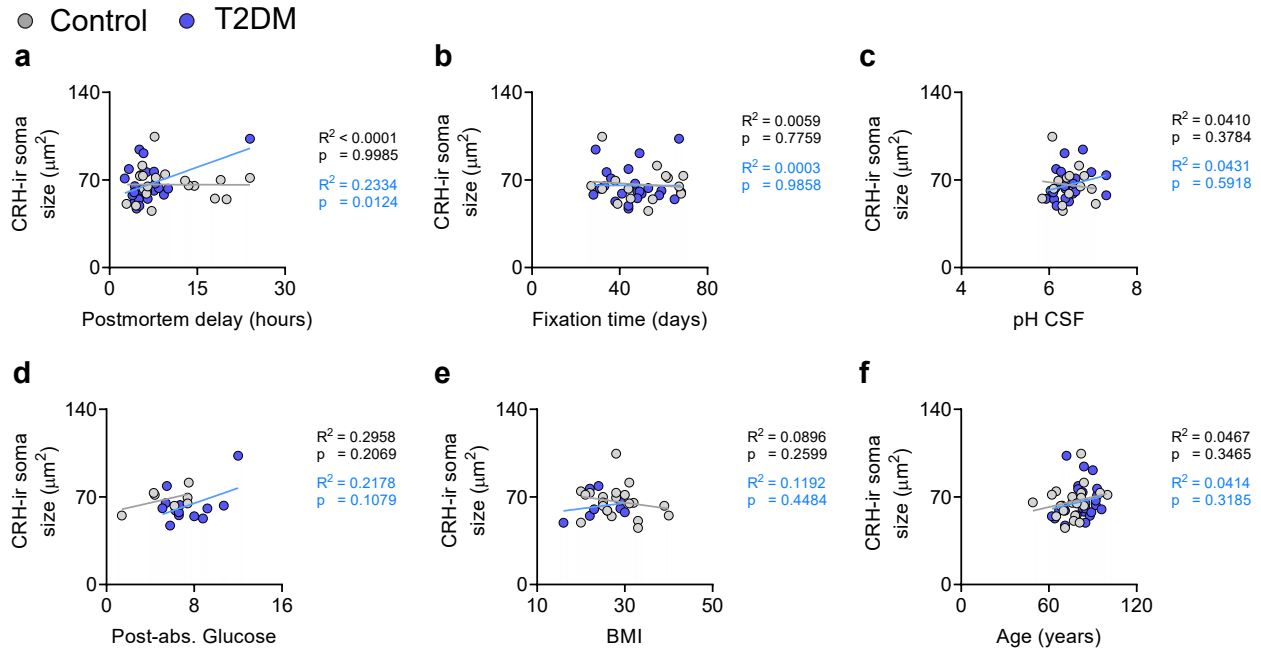
**Supplementary Fig. 16**

**Co-founder analysis of total area masked by positive arginine-vasopressin-ir (AVP-ir) cells in the PVN of control and T2DM subjects.** (a-f) Plots of AVP-ir relative masked area in the PVN according to (a) postmortem delay, (b) fixation time, (c) pH of CSF, (d) post absorptive glucose, (e) and body mass index (BMI) and (f) age. AVP = arginine-vasopressin; PVN = paraventricular nucleus; ir = immunoreactivity; CSF = cerebrospinal fluid; post-abs=post absorptive; T2DM = type 2 diabetic mellitus. Control: n = 20, T2DM: n = 26.



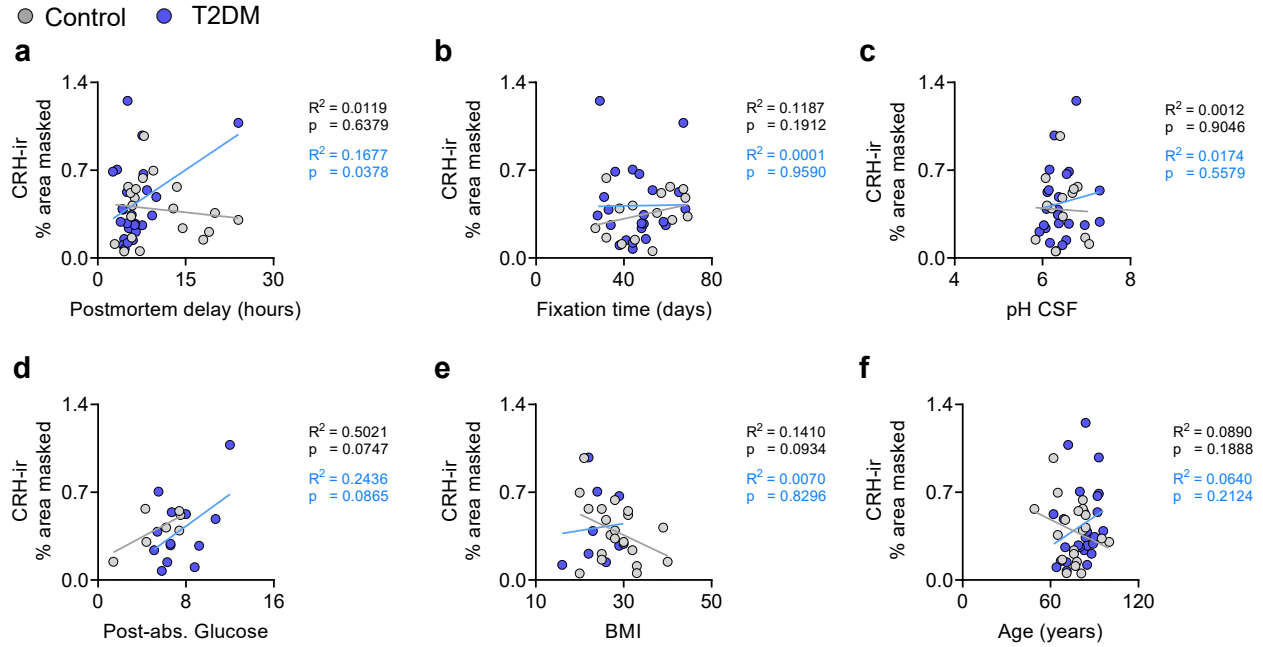
**Supplementary Fig. 17**

**Co-founder analysis of corticotropin releasing hormone-ir (CRH-ir) neurons density in the PVN of control and T2DM subjects.** (a-f) Plots of CRH-ir soma number/mm<sup>2</sup> in the hypothalamic PVN according to (a) postmortem delay, (b) fixation time, (c) pH of CSF, (d) post absorptive glucose, (e) and body mass index (BMI) and (f) age. CRH = corticotropin releasing hormone; PVN = paraventricular nucleus; ir = immunoreactivity; CSF = cerebrospinal fluid; post-abs=post absorptive; T2DM = type 2 diabetic mellitus. Control: n = 20, T2DM: n = 26.



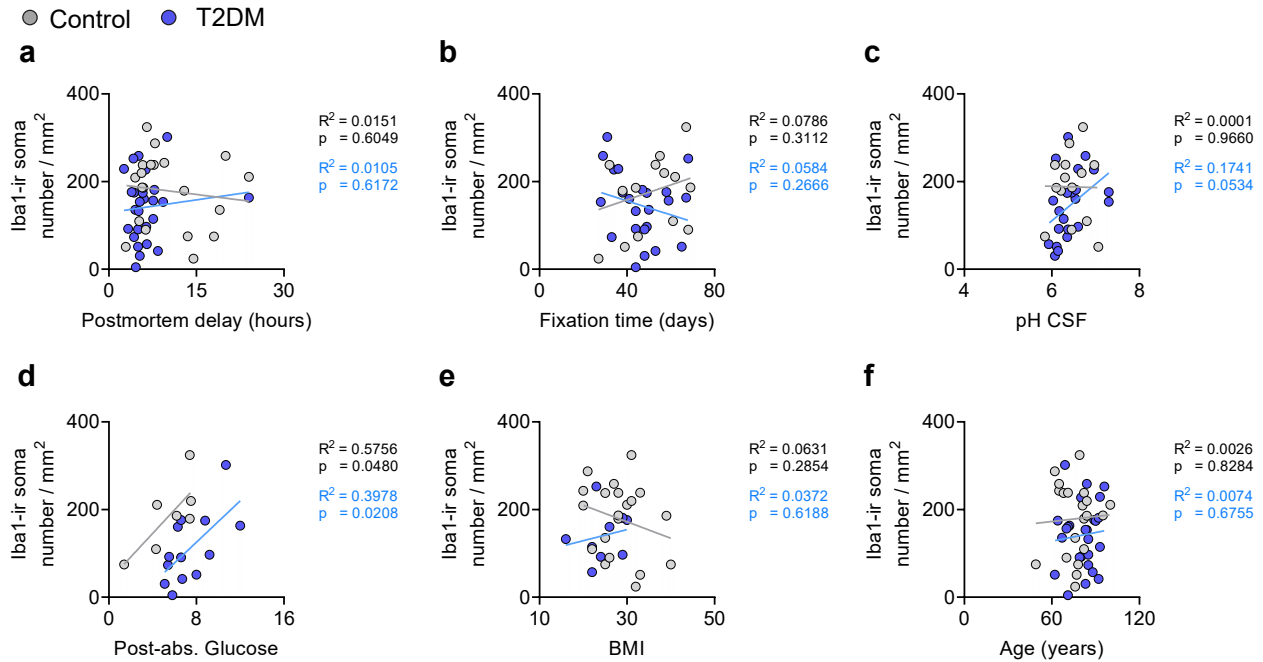
**Supplementary Fig. 18**

**Co-founder analysis of corticotropin releasing hormone-ir (CRH-ir) soma size in the PVN of control and T2DM subjects.** (a-f) Plots of CRH-ir soma size in the PVN according to (a) postmortem delay, (b) fixation time, (c) pH of CSF, (d) post absorptive glucose, (e) and body mass index (BMI) and (f) age. CRH = corticotropin releasing hormone; PVN = paraventricular nucleus; ir = immunoreactivity; CSF = cerebrospinal fluid; post-abs=post absorptive; T2DM = type 2 diabetic mellitus. Control: n = 20, T2DM: n = 26.



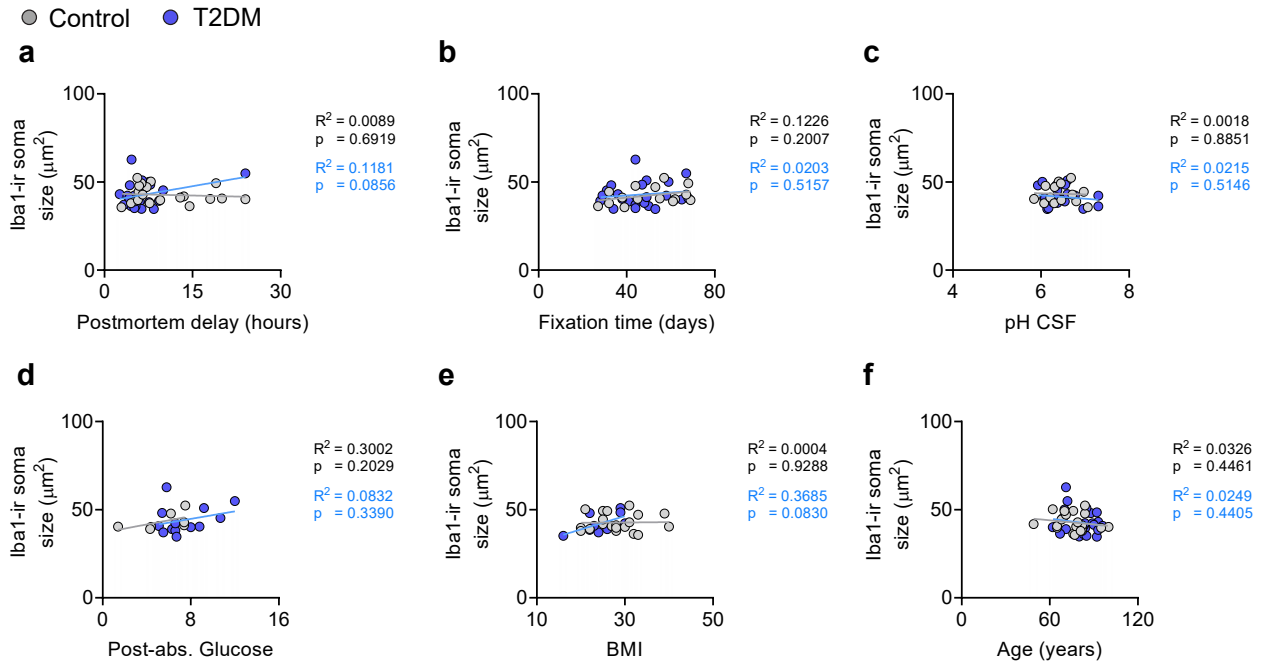
**Supplementary Fig. 19**

**Co-founder analysis of total area masked by positive corticotropin releasing hormone-ir (CRH-ir) cells in the PVN of control and T2DM subjects. (a-f) Plots of CRH-ir relative masked area in the PVN according to (a) postmortem delay, (b) fixation time, (c) pH of CSF, (d) post absorptive glucose, (e) and body mass index (BMI) and (f) age. CRH = corticotropin releasing hormone; PVN = paraventricular nucleus; ir = immunoreactivity; CSF = cerebrospinal fluid; post-abs=post absorptive; T2DM = type 2 diabetic mellitus. Control: n = 20, T2DM: n = 26.**



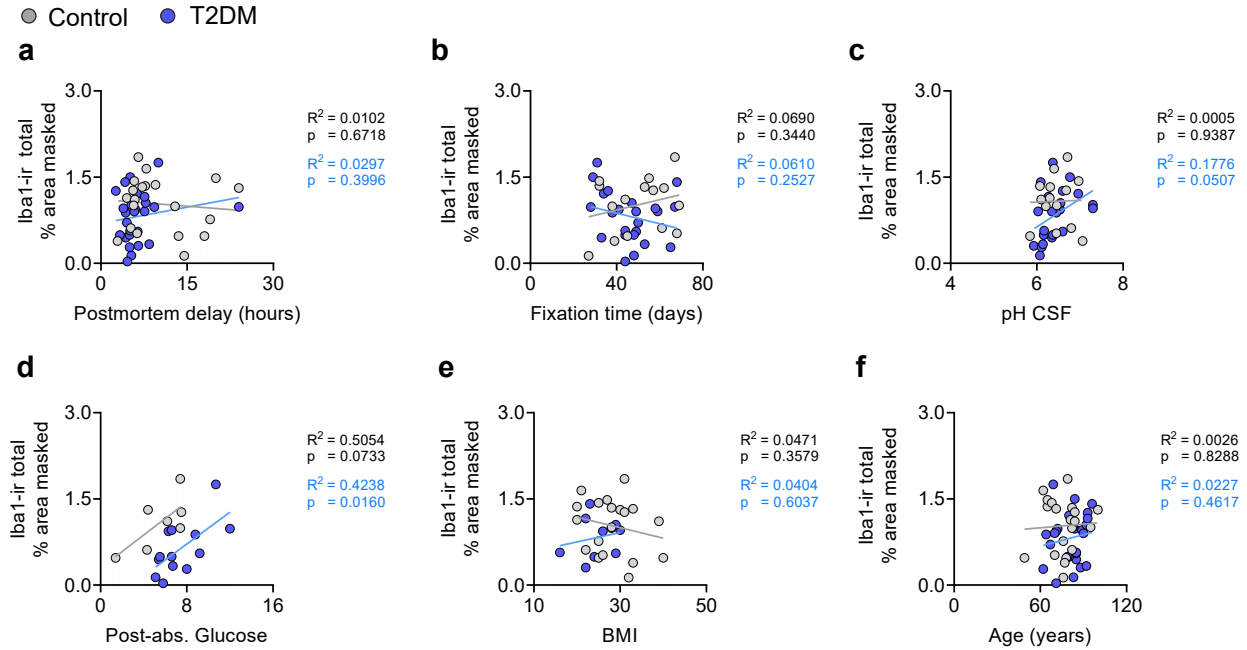
**Supplementary Fig. 20**

**Co-founder analysis of microglial (Iba1-ir) density in the PVN of control and T2DM subjects.** (a-f) Plots of Iba1-ir soma number/mm<sup>2</sup> in the PVN according to (a) postmortem delay, (b) fixation time, (c) pH of CSF, (d) post absorptive glucose, (e) and body mass index (BMI) and (f) age. Iba1 = ionized calcium-binding adapter molecule 1; ir = immunoreactivity; CSF = cerebrospinal fluid; post-abs=post absorptive; T2DM = type 2 diabetic mellitus. Control: n = 20, T2DM: n = 26.



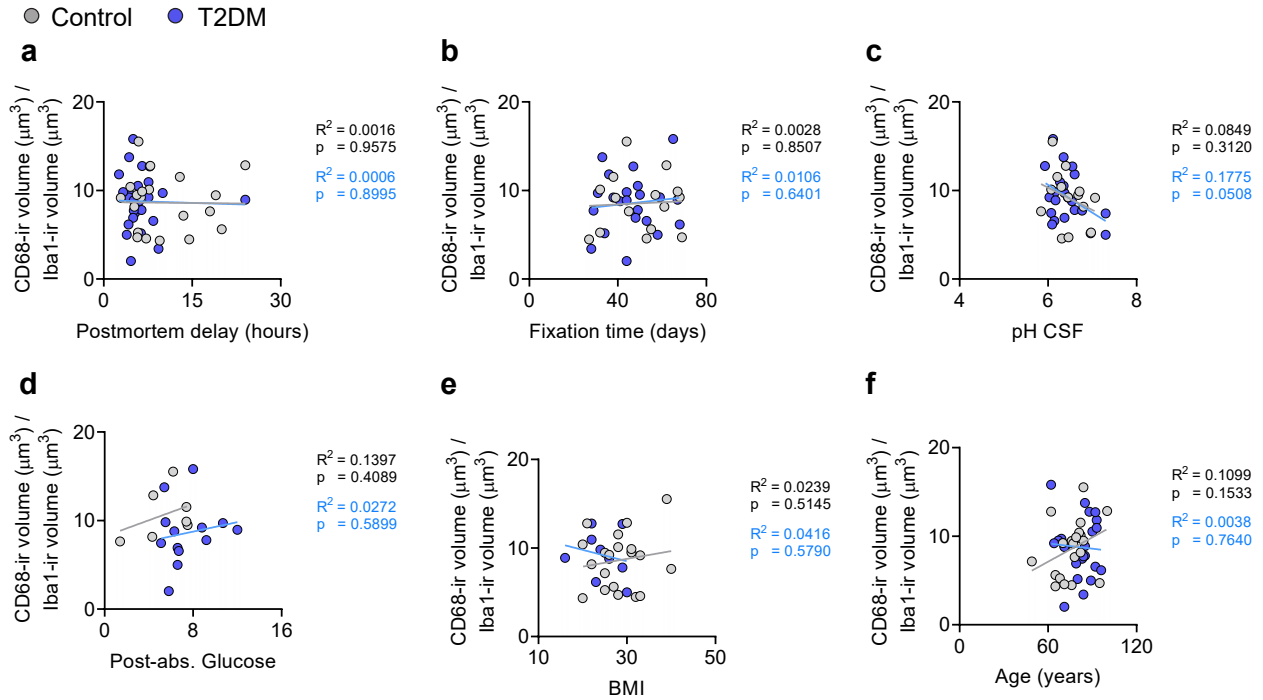
**Supplementary Fig. 21**

**Co-founder analysis of microglial (Iba1-ir) soma size in the PVN of control and T2DM subjects.** (a-f) Plots of Iba1-ir soma size in the PVN according to (a) postmortem delay, (b) fixation time, (c) pH of CSF, (d) post absorptive glucose, (e) and body mass index (BMI) and (f) age. Iba1 = ionized calcium-binding adapter molecule 1; PVN = paraventricular nucleus; ir = immunoreactivity; CSF = cerebrospinal fluid; post-abs=post absorptive; T2DM = type 2 diabetic mellitus. Control: n = 20, T2DM: n = 26.



**Supplementary Fig. 22**

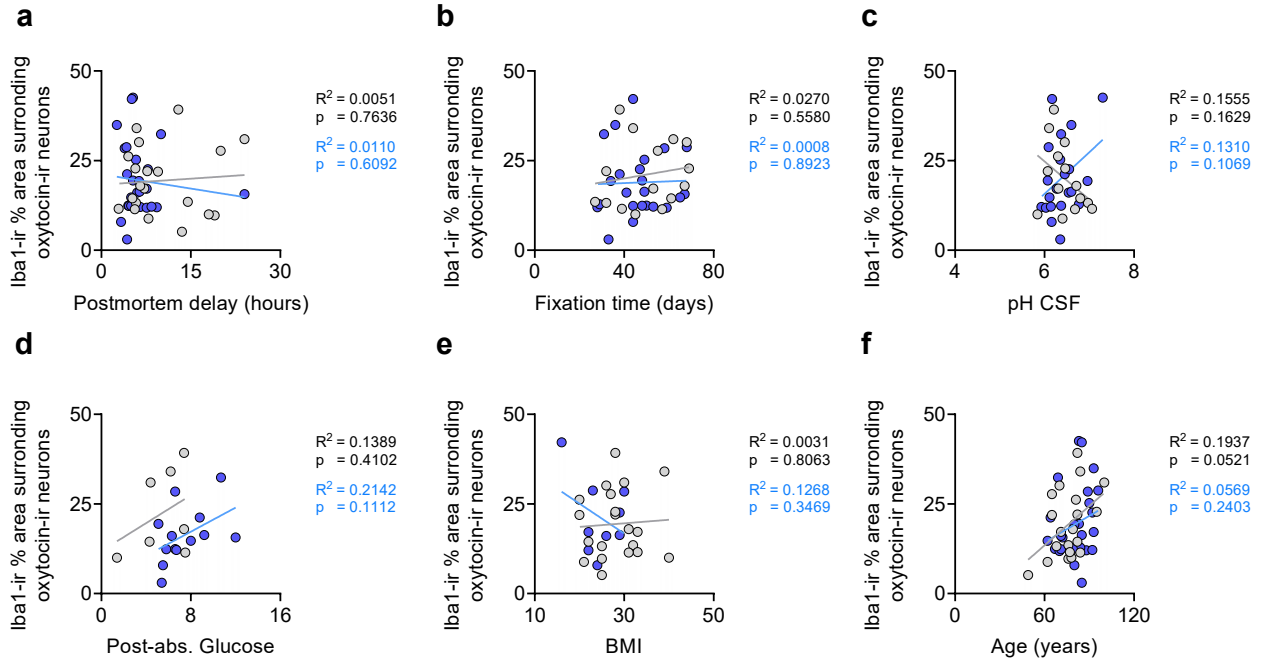
**Co-founder analysis of total area masked by Iba1-ir microglial cells in the PVN of control and T2DM subjects.**  
 (a-f) Plots of Iba1-ir relative masked area in the PVN according to (a) postmortem delay, (b) fixation time, (c) pH of CSF, (d) post absorptive glucose, (e) and body mass index (BMI) and (f) age. Iba1 = ionized calcium-binding adapter molecule 1; PVN = paraventricular nucleus; ir = immunoreactivity; CSF = cerebrospinal fluid; post-abs=post absorptive; T2DM = type 2 diabetic mellitus. Control: n = 20, T2DM: n = 26.



**Supplementary Fig. 23**

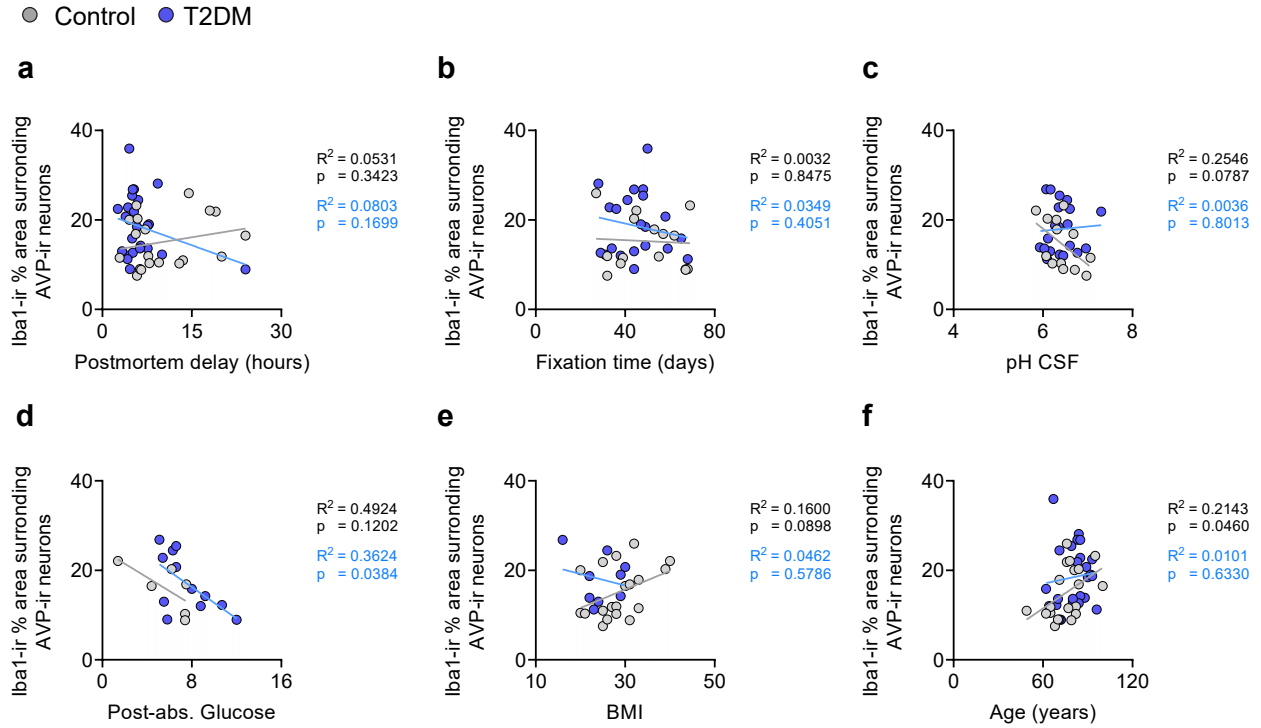
**Co-founder analysis of microglial cluster of differentiation-ir (CD68-ir) in the PVN of control and T2DM subjects.** (a-f) Plots of volume percentage of CD68-ir in relation to Iba1-ir in the PVN according to (a) postmortem delay, (b) fixation time, (c) pH of CSF, (d) post absorptive glucose, (e) and body mass index (BMI) and (f) age. CD68 = cluster of differentiation 68; Iba1 = ionized calcium-binding adapter molecule 1; PVN = paraventricular nucleus; ir = immunoreactivity; CSF = cerebrospinal fluid; post-abs=post absorptive; T2DM = type 2 diabetic mellitus. Control: n = 20, T2DM: n = 26.

● Control ● T2DM



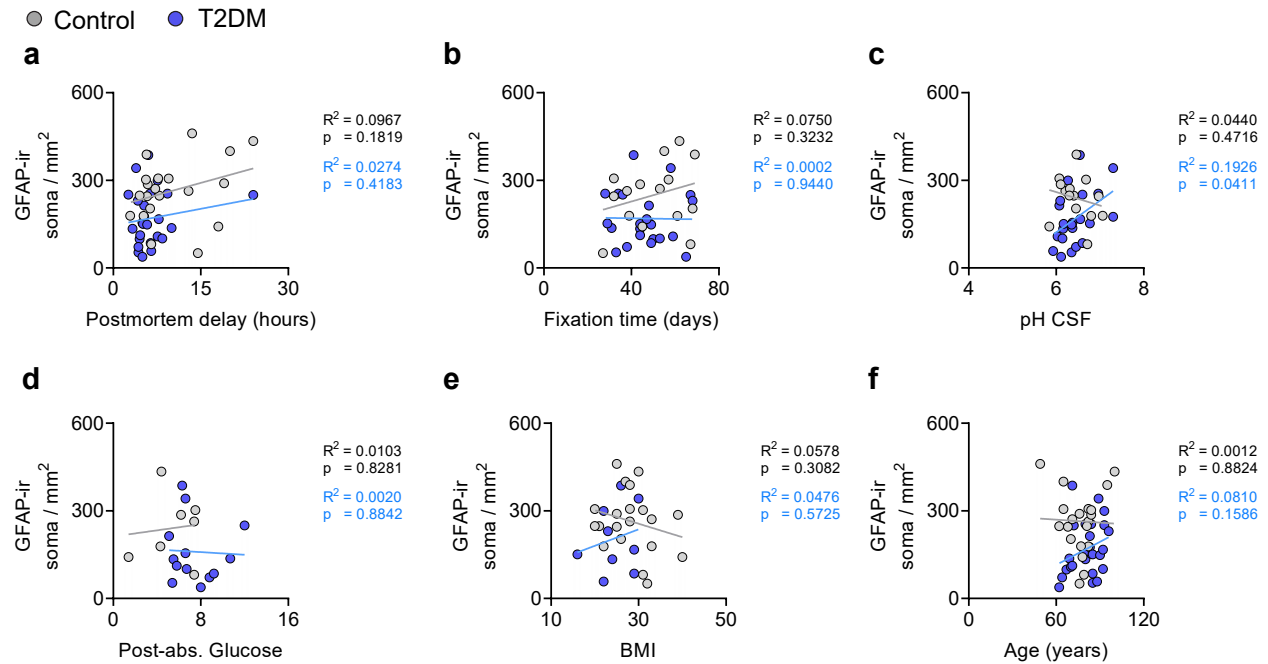
**Supplementary Fig. 24**

**Co-founder analysis of mean relative area masked (%) of Iba1-ir particles surrounding oxytocin-ir (Oxt-ir) neurons in the PVN of control and T2DM subjects.** (a-f) Plots of Iba1-ir relative masked area surrounding Oxt-ir neurons (within 10  $\mu$ m radius) in the PVN according to (a) postmortem delay, (b) fixation time, (c) pH of CSF, (d) post absorptive glucose, (e) and body mass index (BMI) and (f) age. (Iba1 = ionized calcium-binding adapter molecule 1; Oxt = oxytocin; ir = immunoreactivity; CSF = cerebrospinal fluid; post-abs=post absorptive; T2DM = type 2 diabetic mellitus. Control: n = 20, T2DM: n = 26.



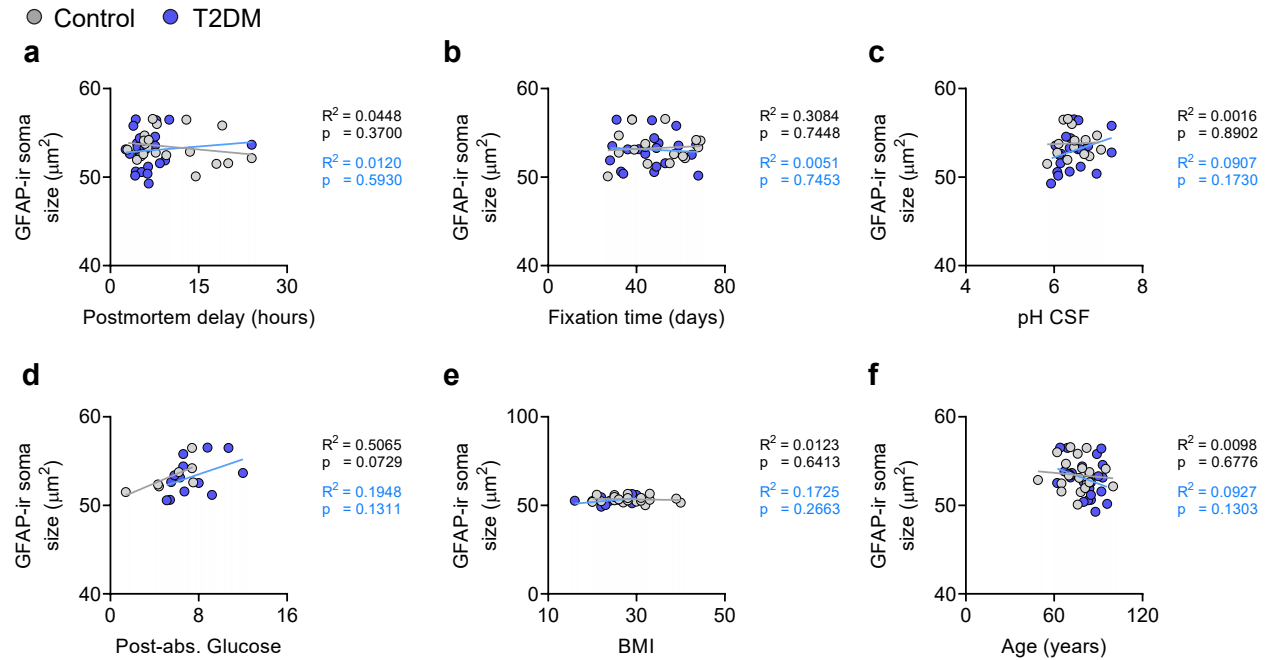
**Supplementary Fig. 25**

**Co-founder analysis of mean relative area masked (%) of Iba1-ir particles surrounding AVP-ir neurons in the PVN of control and T2DM subjects.** (a-f) Plots of Iba1-ir relative masked area surrounding AVP-ir neurons (within 10  $\mu$ m radius) in the PVN according to (a) postmortem delay, (b) fixation time, (c) pH of CSF, (d) post absorptive glucose, (e) and body mass index (BMI) and (f) age. Iba1 = ionized calcium-binding adapter molecule 1; AVP = arginine-vasopressin; PVN = paraventricular nucleus; ir = immunoreactivity; CSF = cerebrospinal fluid; post-abs=post absorptive; T2DM = type 2 diabetic mellitus. Control: n = 20, T2DM: n = 26.



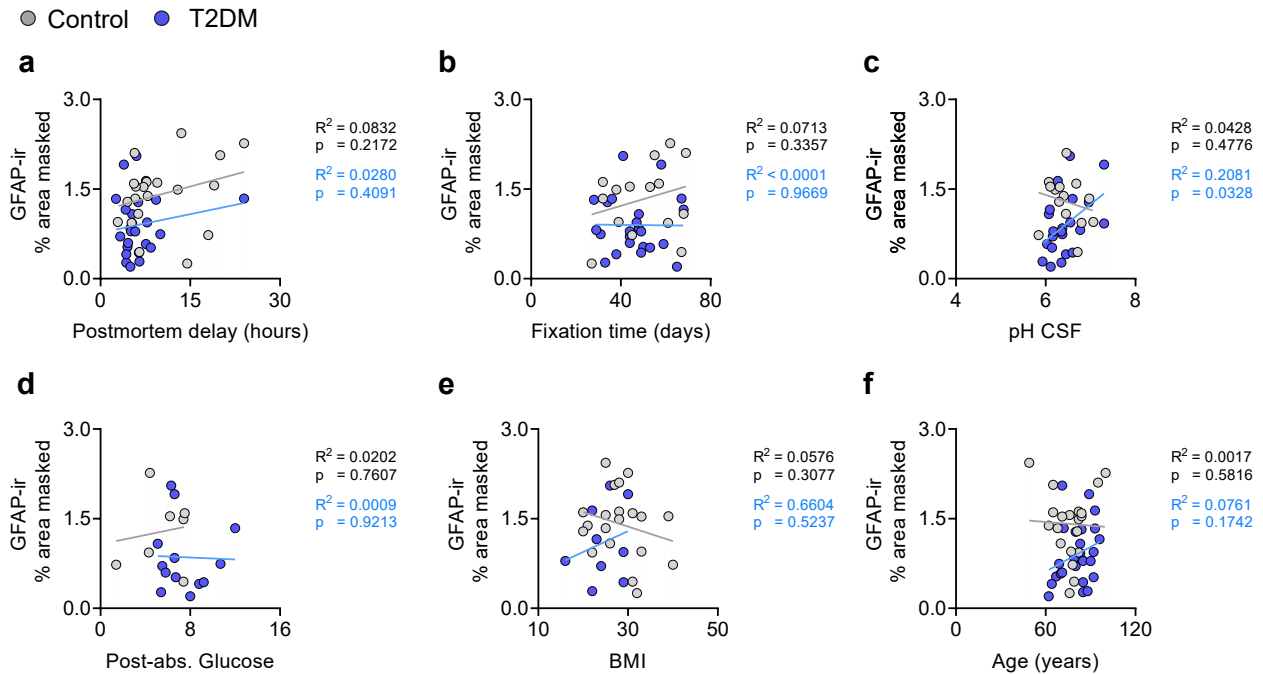
**Supplementary Fig. 26**

**Co-founder analysis of astroglial (GFAP-ir) density in the PVN of control and T2DM subjects. (a-f)** Plots of GFAP-ir soma number/mm<sup>2</sup> in the PVN according to (a) postmortem delay, (b) fixation time, (c) pH of CSF, (d) post absorptive glucose, (e) and body mass index (BMI) and (f) age. GFAP = glial fibrillary acid protein; ir = immunoreactivity; PVN = paraventricular nucleus; CSF = cerebrospinal fluid; post-abs=post absorptive; T2DM = type 2 diabetic mellitus. Control: n = 20, T2DM: n = 26.



**Supplementary Fig. 27**

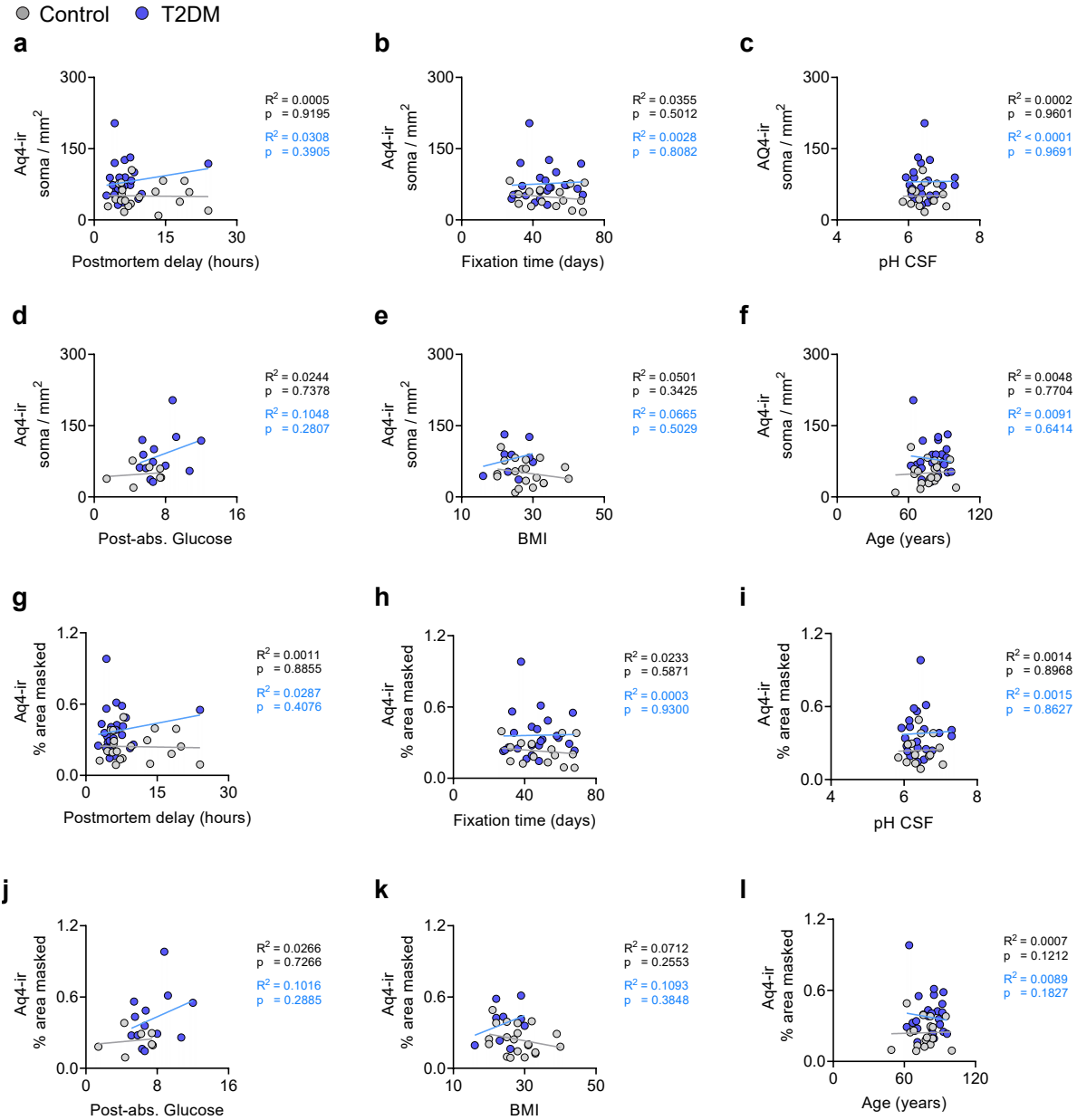
**Co-founder analysis of astroglial (GFAP-ir) soma size in the PVN of control and T2DM subjects.** (a-f) Plots of GFAP-ir soma size in the PVN according to (a) postmortem delay, (b) fixation time, (c) pH of CSF, (d) post absorptive glucose, (e) and body mass index (BMI) and (f) age. GFAP = glial fibrillary acid protein; ir = immunoreactivity; PVN = paraventricular nucleus; CSF = cerebrospinal fluid; post-abs=post absorptive; T2DM = type 2 diabetic mellitus. Control: n = 20, T2DM: n = 26.



**Supplementary Fig. 28**

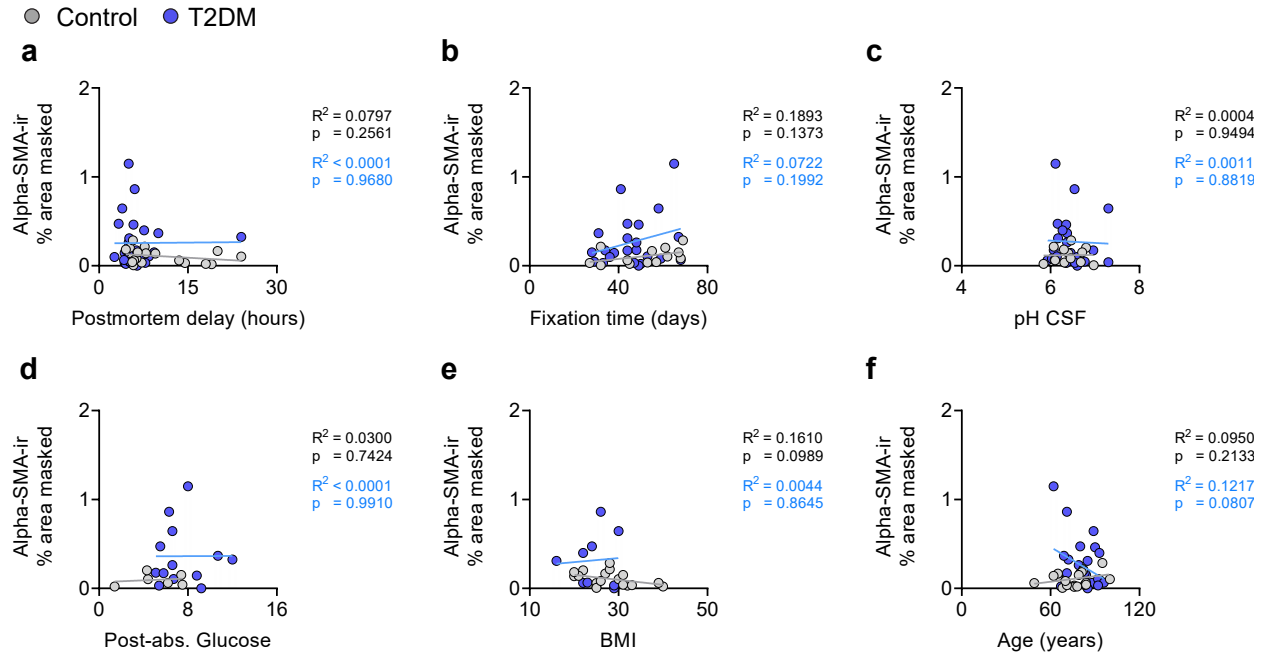
**Co-founder analysis of astroglial (GFAP-ir) relative masked area in the PVN of control and T2DM subjects.**

(a-f) Plots of GFAP-ir relative masked area in the PVN according to (a) postmortem delay, (b) fixation time, (c) pH of CSF, (d) post absorptive glucose, (e) and body mass index (BMI) and (f) age. GFAP = glial acid fibrillary acid protein; PVN = paraventricular nucleus; ir = immunoreactivity; CSF = cerebrospinal fluid; post-abs=post absorptive; T2DM = type 2 diabetic mellitus. Control: n = 20, T2DM: n = 26.



**Supplementary Fig. 29**

**Co-founder analysis of aquaporin 4-ir astrocytes (Aq4-ir) in the PVN of control and T2DM subjects.** (a-f) Plots of Aq4-ir soma number/mm<sup>2</sup> in the PVN according to (a) postmortem delay, (b) fixation time, (c) pH of CSF, (d) post absorptive glucose, (e) and body mass index (BMI) and (f) age. (g-l) Plots of Aq4-ir relative masked area in PVN according to (g) postmortem delay, (h) fixation time, (i) pH of CSF, (j) post absorptive glucose, (k) and body mass index (BMI) and (l) age. Aq4 = aquaporin 4; PVN = paraventricular nucleus; ir = immunoreactivity; CSF = cerebrospinal fluid; post-abs=post absorptive; T2DM = type 2 diabetic mellitus. Control: n = 20, T2DM: n = 26.



**Supplementary Fig. 30**

**Co-founder analysis of vasculature (alpha-SMA-ir) relative masked area in the PVN of control and T2DM subjects.** (a-f) Plots of alpha-SMA-ir relative masked area in the PVN according to (a) postmortem delay, (b) fixation time, (c) pH of CSF, (d) post absorptive glucose, (e) and body mass index (BMI) and (f) age.  $\alpha$ SMA = alpha smooth muscle actin; PVN = paraventricular nucleus; ir = immunoreactivity; CSF = cerebrospinal fluid; post-abs=post absorptive; T2DM = type 2 diabetic mellitus. Control: n = 20, T2DM: n = 26.