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Abstract: Major depression is a highly prevalent severe mood disorder that is treated with antidepressants. The molecular targets of antidepressants require definition. We investigated the role of the acid sphingomyelinase (Asm)-ceramide system as a target for antidepressants. Therapeutic concentrations of the antidepressants amitriptyline and fluoxetine reduced Asm activity and ceramide concentrations in the hippocampus, increased neuronal proliferation, maturation and survival and improved behavior in mouse models of stress-induced depression. Genetic Asm deficiency abrogated these effects. Mice overexpressing Asm, heterozygous for acid ceramidase, treated with blockers of ceramide metabolism or directly injected with C16 ceramide in the hippocampus had higher ceramide concentrations and lower rates of neuronal proliferation, maturation and survival compared with controls and showed depression-like behavior even in the absence of stress. The decrease of ceramide abundance achieved by antidepressant-mediated inhibition of Asm normalized these effects. Lowering ceramide abundance may thus be a central goal for the future development of antidepressants.

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Acid sphingomyelinase/ceramide system mediates effects of antidepressant drugs

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Abstract

Major depression is a highly prevalent severe mood disorder that is treated with antidepressant medications[1]. The molecular targets of antidepressants require definition. We tested the role of the acid sphingomyelinase (Asm)/ceramide system as target for antidepressants. Therapeutic concentrations of the antidepressants, amitriptyline and fluoxetine, reduce Asm activity and ceramide concentrations in the hippocampus, increase neuronal proliferation, maturation, and survival, and improve behavior in mouse models of stress-induced depression. Genetic Asm deficiency abrogates these effects of antidepressants. Mice overexpressing Asm, heterozygous for acid ceramidase, treated with blockers of ceramide metabolism, or directly injected with C16 ceramide in the hippocampus exhibit increased ceramide concentrations, decreased neuronal proliferation, maturation, and survival, and depression-like behavior even in the absence of stress. All these features are normalized by the decrease of ceramide levels via antidepressant-mediated inhibition of Asm. Lowering ceramide levels may thus be a central goal for the future development of novel antidepressants.

Major depression is a severe, chronic, and often life-threatening illness with a lifetime prevalence of more than 10%[1]. Key symptoms of depression include depressed mood, loss of interest and pleasure, feelings of worthlessness, weight loss, insomnia and at least 10% of patients commit suicide.

Major depression may be triggered by psychological stress, inflammatory cytokines, and dysfunction of the hypothalamic-pituitary-adrenal axis, etc.[1–4].

The previously held monoamine hypothesis for the action of antidepressants has been questioned because the antidepressant effect of these drugs is not clearly associated with their monoaminergic effect; in fact, the antidepressant tianeptine is even a serotonin reuptake enhancer[5]. Furthermore, the direct effect on monoamines contrasts with the delay of antidepressant effects in patients. Recent concepts of the pathogenesis of major depression suggest a change of cellular plasticity predominantly in the hippocampus and a shift in the balance between neurogenic and antiapoptotic events that leads to neurodegeneration and hippocampal atrophy[6–9]. Antidepressants increase neurogenesis and reverse hippocampal atrophy associated with major depression[9].

Here, we tested the role of the acid sphingomyelinase (EC 3.1.4.12, sphingomyelin phosphodiesterase, human protein: ASM, murine protein: Asm, gene symbol: *Smpd1*) and ceramide system as a target for antidepressants. Asm is ubiquitously expressed and releases ceramide from sphingomyelin, predominantly in lysosomes but also in secretory lysosomes and on the plasma membrane[10–13].

The antidepressants amitriptyline, a tricyclic drug, and fluoxetine, a selective serotonin reuptake inhibitor, inhibited ASM activity in cultured neurons at therapeutic plasma concentrations recommended for patients with major depression[14] (**Fig. 1a**). Treatment of wild-type (WT) mice with amitriptyline or fluoxetine dose dependently reduced hippocampal Asm activity and protein levels (**Fig. 1b,c**; **Supplementary Fig. 1a,b,d, Supplementary note 1**), a finding consistent with the induction of partial proteolysis of Asm by amitriptyline and fluoxetine[15,16]. Mice transgenic for Asm (t-Asm) exhibited increased Asm activity and protein levels in the hippocampus, and amitriptyline and fluoxetine reduced Asm activity and protein levels (**Fig. 1b; Supplementary Fig. 1a,c,d**). Asm-deficient mice were used as specificity control (**Supplementary Fig. 1a).**

The reduction of Asm levels and activity by amitriptyline or fluoxetine translated into a dose-dependent reduction of ceramide in the hippocampus of WT and t-Asm mice, as determined by diacylglycerol (DAG) kinase assays (**Fig. 1d**), mass spectrometry (**Supplementary Fig. 2a**) and fluorescence microscopy (**Fig. 1e**; **Supplementary Fig. 2b–e,h**). In contrast, the antidepressants did not alter hippocampal ceramide levels in Asm-deficient mice (**Fig. 1d**; **Supplementary Fig. 2a–c,f,h**). Constitutive hippocampal ceramide concentrations were lower in Asm-deficient mice, and were higher in t-Asm mice, compared with WT mice (**Fig. 1d**; **Supplementary Fig. 2a–c**).

To induce higher constitutive hippocampal ceramide independent of Asm, we generated acid ceramidase (Ac)-heterozygous mice, which exhibited approximately 50% loss of Ac activity (data not shown) and very high concentrations of ceramide in the hippocampus; these concentrations were reduced by antidepressants (**Fig. 1d**, **Supplementary Fig. 2a–c,g,h**).

Because major depression has been linked to stress[1], we tested the effects of corticosterone, a well-established stress inducer (**Supplementary note 2**) on Asm and ceramide. Corticosterone did not affect Asm activity or expression or ceramide levels (**Fig. 1b,d; Supplementary Fig. 2a,i–n)**.

Next, we determined whether inhibition of Asm mediates effects of amitriptyline and fluoxetine on neurogenesis, neuronal maturation and survival. Neurogenesis, neuronal maturation, and neuronal survival were lower in t-Asm and Ac-heterozygous mice than in WT mice, but were higher in Asm-deficient mice than in WT mice even in the absence of any treatment (**Fig. 2a–c**, **Supplementary Figs. 3a–d**). Amitriptyline and fluoxetine increased neurogenesis, neuronal maturation, and survival in the hippocampus of stressed or non-stressed WT, t-Asm, and Ac-heterozygous mice, but not in Asm-deficient mice (**Fig. 2a–c**, **Supplementary Fig. 3a**). Controls show that hippocampal structural synaptic parameters and electrophysiological properties of hippocampal neurons did not differ between WT, Asm-deficient and t-Asm mice or cells, respectively (**Supplementary Fig. 4a–d**). Ceramide reduced Akt phosphorylation at serine 473, which was increased by antidepressants (**Fig. 2d**). C16 ceramide also inhibited proliferation of PC12 cells, an effect that was abrogated by expression of the constitutively-active T308DS473DAkt1 mutant of Akt1 (**Supplementary Fig. 5**).

The hypothesis that the Asm/ceramide system plays a role in the effect of antidepressants was confirmed by the abrogation of the previously-described effect of fluoxetine on egg laying[17,18], which was abrogated in *ams*-1-deficient *C. elegans* (**Fig. 2e**). 5-HT still induced egg laying in *asm*-1-deficient worms and deletion of Mod-5, the only serotonin transporter expressed in *C. elegans*, did not alter the effects of fluoxetine indicating that the 5-HT system is independent or at least partially downstream of *asm*-1 (**Fig. 2e**; **Supplementary Figs. 6,7a,b**).

To further substantiate the role of the Asm/ceramide system as a target for antidepressants, we tested whether fendiline, a functional inhibitor of ASM[16], that has not been reported to be an antidepressant, also acts as antidepressant. Fendiline reduced Asm activity and reduced ceramide concentrations in the hippocampus in WT and t-Asm (**Fig. 2f; Supplementary Fig. 8a, Supplementary note 3**). This resulted in increased neurogenesis, neuronal maturation and survival in WT, but not Asm-deficient mice, very similar to amitriptyline and fluoxetine (**Fig. 2g; Supplementary Fig. 8b-d, Supplementary note 3**).

To further investigate the role of Asm in major depression, we determined whether ceramide determines the effects of amitriptyline, fluoxetine and fendiline on mouse behavior.

Increased ceramide levels in the hippocampus of t-Asm mice and Ac-heterozygous mice were associated with depression-like behavior (**Fig. 3a–f**; **Supplementary Fig. 9**). Treatment with antidepressants or fendiline normalized this endogenous stress-like state. *Vice versa*, Asm-deficient mice showed constitutively reduced stress behavior and, importantly, antidepressants or fendiline had no effect on Asm-deficient mice. Corticosterone application resulted in severe depressive-like behavior in all mouse types (**Fig. 3a–f**; **Supplementary Fig. 9**). Treatment with antidepressants or fendiline attenuated the corticosterone-induced depressive-like behavior in WT, t-Asm, and Ac-heterozygous mice, respectively, but had no effect on corticosterone-induced depressive-like behavior in Asm-deficient mice. These findings indicate that several behavioral effects of antidepressants depend on the presence of Asm and are mediated by a reduction of ceramide concentrations.

To further address the role of ceramide versus Asm in major depression, we increased ceramide in the hippocampus independent of Asm and Ac by administering the glycosyltransferase inhibitor DL-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP), which prevents glycosylation of ceramide and has been shown to increase ceramide concentrations in gerbil hippocampus and cells[19,20]. PDMP treatment doubled ceramide concentrations, decreased neurogenesis and neuronal maturation in the hippocampus and induced depression-like symptoms in WT and Asm-deficient mice (**Fig. 4a,b**; **Supplementary Fig. 10a–g**). These alterations were corrected in WT mice by treatment with amitriptyline or fluoxetine, while the antidepressants had no effects in Asm-deficient mice treated with PDMP (**Fig. 4a,b**; **Supplementary Fig. 10a–g**).

Further, reduction of ceramide in Ac-heterozygous mice by reduction of gene dosis of Asm normalized neurogenesis, maturation and depression-like behavior (**Fig. 4a,b**; **Supplementary Fig. 10a–g**).

Finally, we injected C16 ceramide directly into the hippocampus of WT mice, which induced depression-like symptoms, while solvent controls were without effect (**Fig. 4c**; **Supplementary Fig. 10h**).

To test the role of the Asm/ceramide system for the effects of antidepressants in a different stress model, we used the chronic unpredictable stress model. Chronic unpredictable stress increased ceramide concentrations ~2-fold in the hippocampus of both WT and Asm-deficient mice and concomitantly reduced neurogenesis and maturation (**Fig. 4d,e**), events that were normalized by antidepressants in WT mice, but not in Asm-deficient mice.

Major depression is a common, complex, likely multifactorial psychiatric disorder. Here we propose a novel mode of action for antidepressants (**Supplementary Fig. 11**): The complex balance between positive and negative stimuli in the hippocampus determines development of major depression. Ceramide acts as a negative regulator in the multifactorial pathogenesis of major depression. Inhibiting Asm activity reduces hippocampal ceramide concentrations and, thus, reduces the sum of the negative stimuli, thereby permitting normalization of behavior, neurogenesis, neuronal maturation, and neuronal survival.

Ceramide may regulate neurogenesis via several pathways: Ceramide controls activity of Akt shown to be important for neuronal proliferation[21]. Ceramide has been also shown to regulate the formation of reactive oxygen species via activation of NADPH oxidases[22], to mediate the effects of the cytokines interleukin (IL)-1β and tumor necrosis factor (TNF)-α[23–25] and to regulate stress-activated kinases[26] and calcium release[27]. Thus, ceramide may coordinate negative effects on hippocampal neurons by Akt inhibition and assembling of molecules involved in oxidative stress, proinflammatory receptors and stress-signaling. Antidepressants counteract these events by reducing ceramide levels. This view is consistent with the finding that patients with major depression experience a wide range of abnormalities in organs such as heart, bone, and blood, in which oxidative stress, proinflammatory cytokines and sphingolipid levels are elevated[28–32]. These pathologic conditions might be explained by the increased ceramide concentration in major depression, not only in hippocampal neurons but also in other cells[33].

We therefore propose that ASM is an important target of antidepressant drugs in the hippocampus. We suggest that cellular ceramide concentrations may be a novel intermediate phenotype controlling hippocampal functions, including neurogenesis and neuronal networks that determine behavior.

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Author contributions: E.G. and J.K. initiated the studies, designed experiments, supervised research, and wrote the manuscript. E.G. also performed most animal studies. E.G., K.A.B., and G.T. performed the histological studies and developed the polyclonal anti-Asm antibody. M.P. and C.B. performed the *C. elegans* studies. A.L. and B.K. measured ceramide concentrations by mass spectrometry. M.W. designed

some experiments and participated in BrdU stainings. H.G. performed the confocal microscopy studies. J.K., P.T. and S.St. performed experiments on the concentration-dependent inhibition of ASM by antidepressant drugs. M.R. and M.P. performed experiments on 5-HT uptake in cultured hippocampal neurons. M.R. and J.K. performed experiments on 5-HT uptake in mouse brain synaptosomes. C.H.T. and T.W.G. designed and performed synapse staining and confocal analyses. T.F.A, U.E.L., and E.G. performed behavioral experiments. C.P.M, D.A., M.R. and J.K. designed and performed hippocampal injection and microdialysis studies. C.A., J.v.B., M.R. and J.K. designed and performed electrophysiological studies in hippocampal slices. All authors discussed the results and commented on the manuscript.

The authors declare to have no competing financial interests.

Methods

Mice and treatments: Asm-deficient mice (*Smpd1*$^{-/-}$) show age-dependent accumulation of sphingomyelin and development of Niemann-Pick syndrome A or B, depending on the residual activity of ASM[34]. To avoid sphingomyelin accumulation, we used only *Smpd1*$^{-/-}$ mice with a maximum age of 12 weeks[35]. We observed no increase in cell death in the hippocampus of these mice (not shown). In Asm transgenic mice, the murine *Smpd1* cDNA was expressed under the control of the ubiquitous CMV immediate-early enhancer/chicken β-actin promoter fusion promoter. A loxP-flanked STOP cassette was included between the promoter and the transgene so that the expression could be conditionally regulated by the action of Cre recombinase. The conditional transgene was introduced into the deleted Hprt gene locus of E14 embryonic stem cells. The transgenic mice were generated and back-crossed for at least 5 generations to C57BL/6 mice. The transgene was constitutively expressed by crossing these mice with mice expressing Cre recombinase under the control of an E2A promoter[36]. For acid ceramidase 1 (*Asah1*)-heterozygous mice, the exon/intron organization of the gene was established on the basis of the *Asah1* cDNA sequence NM_019734. A targeting vector containing regions homologous to genomic *Asah1* sequences was constructed. It included homology regions in the C57BL/6J genetic background, a long homology region of 5.8 kb and a short homology region of 1.7 kb; two loxP sites flanking *Asah1* exon 1; a neomycin gene flanked by flippase recognition target sites for positive selection; and a diphtheria toxin A negative-selection marker to reduce the isolation of non-homologous recombined embryonic stem (ES) cell clones and to enhance the isolation of ES cell clones harboring the distal loxP site. This targeting vector was inserted into C57BL/6 embryonic stem cells by homologous recombination. Offspring were crossed with E2A-Cre mice to generate mice heterozygous for Ac.

Amitriptyline (100, 140, 180, or 220 mg L^{-1}), fluoxetine (40, 80, 120 or 160 mg L^{-1}), or fendiline (17, 33, 67.5, or 135 mg L^{-1}) were administered for 35 days to mice via their drinking water. Drugs were dissolved in water, which was changed every 48 h. BrdU was injected at a dose of 75 mg kg^{-1} 4 times (every 2 h) either 1 day or 21 to 28 days before the mice were sacrificed. Corticosterone was administered at 0.25 mg mL^{-1} in the drinking water for 28 days. If amitriptyline, fluoxetine, and corticosterone were administered together, the corticosterone treatment was initiated 7 days after the initiation of antidepressant treatment.

In the chronic unpredictable stress model the mice were challenged with unpredictable environmental stress for 5 weeks, i.e shift of the day-light cycle (for light/dark successions of 30 min every 24 h once a week), reversal of the light/dark cycle once a week, 3 h of 45 ° tilting of the cage twice a week, water deprivation for 14 h once a week, predator sounds (15 min) three times a week. We used male and female mice. All studies were performed in accordance with animal permissions of the Regierungspraesidium Düsseldorf, Tübingen and the Regierung von Mittelfranken.

Plasma levels of antidepressant drugs: Plasma levels of amitriptyline were measured by high-performance liquid chromatography; those of fluoxetine and fendiline by liquid chromatography tandem mass spectrometry.

Pharmacological inhibition of ASM activity *in vitro*: Human brain neuroglioma H4 cells (Promochem, Wesel, Germany) were cultured in DMEM with 10% fetal bovine serum and 4 mM glutamine at 37 °C, 8.5% CO_2. H4 cells were grown to a confluence of 80% to 90%, incubated with amitriptyline, fluoxetine or fendiline at final concentrations from 0.1 to 10 μM for 24 h, washed and ASM activity was determined in whole-cell lysates[11,16].

Asm activity in mouse hippocampus: The hippocampal area was removed, shock frozen, and lysed in 250 mM sodium acetate (pH 5.0), 1% NP40, and 1.3 mM EDTA for 15 min. The tissues were then homogenized by two rounds of sonication for 10 s each with a tip sonicator. Aliquots of the lysates were diluted to 250 mM sodium acetate (pH 5.0), 0.1% NP40, and 1.3 mM EDTA; incubated with 50 nCi per sample [^{14}C]sphingomyelin for 30 min at 37 °C; and processed as above.

Measurement of ceramide by DAG kinase method: The hippocampal area was removed, homogenized in 200 μL H_2O by tip sonication, and extracted in $CHCl_3$:CH_3OH:1N HCl (100:100:1, v/v/v). The lower phase was collected, dried, resuspended in 20 μL of a detergent solution (7.5% [w/v] n-octyl glucopyranoside, 5 mM cardiolipin in 1 mM diethylenetriaminepentaacetic acid [DTPA]), and sonicated for 10 min. The kinase reaction was started by the addition of 70 μL of a reaction mixture containing 10 μL diacylglycerol (DAG) kinase (GE Healthcare Europe, Munich, Germany), 0.1 M imidazole/HCl (pH 6.6), 0.2 mM DTPA (pH 6.6), 70 mM NaCl, 17 mM $MgCl_2$, 1.4 mM ethylene glycol tetraacetic acid, 2 mM dithiothreitol, 1 μM adenosine triphosphate (ATP), and 5 μCi [^{32}P]γATP. The kinase reaction was performed for 30 min at room temperature and terminated by the addition of 1 mL

$CHCl_3$:CH_3OH:1N HCl (100:100:1, v/v/v), 170 µL buffered saline solution (135 mM NaCl, 1.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5.6 mM glucose, 10 mM HEPES [pH 7.2]), and 30 µL of a 100 mM EDTA solution. The lower phase was collected, dried, and separated on Silica G60 thin-layer chromatography (TLC) plates with chloroform/acetone/methanol/acetic acid/H_2O (50:20:15:10:5, v/v/v/v/v). The TLC plates were exposed to radiography films, the spots were removed from the plates, and the incorporation of [^{32}P] into ceramide was measured by liquid scintillation counting. Ceramide amounts were determined by comparison with a standard curve using C16 to C24 ceramides as substrates.

LC-MS analysis: The hippocampal area was removed and homogenized in 1 mL of methanol by tip sonication. Ceramides were extracted, 20 pmol of C17 ceramide in methanol were added as an internal standard, followed by the addition of 1 mL each of chloroform, distilled water, and methanol. After 2 min of intensive stirring, 1 mL chloroform was added. The sample was stirred again for 2 min, and 1 mL of distilled water was added. The lower organic phase was collected, and the aqueous phase was extracted twice with 1 mL of chloroform. The combined organic phases were dried, lipids were resolved in 200 µL of methanol and analysed by rapid-resolution liquid chromatography mass spectrometry (LC-MS) using an Agilent 1200 Series binary pump, a degasser, and an autosampler (Agilent Technologies, Böblingen, Germany). A quadrupole time-of flight 6530 mass spectrometer equipped with Jet-Stream Technology operating in the positive electrospray ionization mode was used for detection (Agilent Technologies). High-purity nitrogen for the mass spectrometer was produced by a nitrogen generator (Parker Balston, Maidstone, UK). Chromatographic separations were obtained with a ZORBAX Eclipse XDB-C18 (C18, 4.6×50 mm, 1.8 µm particle size, 80 Å pore size; Agilent Technologies). The injection volume per sample was 10 µL. An isocratic solvent system consisting of acetonitrile/2-propanol 3:2 (v/v) with 1% formic acid and a flow rate of 1 mL min^{-1} over 15 min was used. For mass spectrometric measurements, the following ion source conditions and gas settings for positive LC-MS/MS were adjusted: sheath gas temperature, 400 °C; sheath gas flow, 9 L min^{-1}; nebulizer pressure, 30 psig; drying gas temperature, 350 °C; drying gas flow, 8 L min^{-1}; capillary voltage, 2000 V; fragmentor voltage, 355 V; nozzle voltage, 2000 V. All ceramides gave the same fragment ion of *m/z* 264.27 at various retention times, depending on their chain length. Counting was performed with Mass Hunter Software. Calibration curves of

reference ceramides were performed from 1 pmol to 100 pmol and were constructed by linear fitting using the least-squares linear regression calculation. The resulting slope of the calibration curve was used to calculate the concentration of the respective analyte in the samples.

Immunohistochemical analysis of Asm and ceramide: Mice were euthanized and perfused via the left heart for 2 min with 0.9% NaCl and for 15 min with 4% paraformaldehyde (PFA) buffered in PBS (pH 7.3). Brains were removed, fixed for an additional 36 h in 4% buffered PFA in PBS, and embedded in paraffin. The hippocampus was serially sectioned. The sections were dewaxed, incubated for 30 min with pepsin (Digest All, Invitrogen, Darmstadt, Germany) at 37 °C, washed, and blocked for 10 min with PBS, 0.05% Tween 20, and 5% fetal calf serum (FCS). They were then immunostained for 45 min with polyclonal rabbit antibodies against murine Asm, obtained by immunization with a glutathione-S-transferase (GST) fusion protein (aa 518–564) of mouse Asm. The results were confirmed by staining with a monoclonal antibody against Asm. Ceramide was detected with a ceramide-specific antibody (clone S58-9, Glycobiotech, Kükels, Germany). All antibodies were diluted 1:100 in HEPES/Saline (H/S; 132 mM NaCl, 20 mM HEPES [pH 7.4], 5 mM KCl, 1 mM $CaCl_2$, 0.7 mM $MgCl_2$, 0.8 mM $MgSO_4$) + 1% FCS, washed 3 times in PBS + 0.05% Tween 20, incubated for 45 min with Cy3-coupled donkey anti-rabbit IgG or anti-mouse IgM $F(ab)_2$ fragments (Jackson ImmunoResearch, Newmarket, UK), washed again three times in PBS + 0.05% Tween 20 and once in PBS, and embedded in Mowiol. Immunofluorescence was measured with the Leica TCS SL software program, version 2.61 (Leica, Mannheim, Germany).

Immunohistochemical BrdU, doublecortin, NeuN, and GFAP stainings: For BrdU staining, mice were injected with BrdU, sacrificed and brains were prepared as above. Paraffin-embedded sections were dewaxed, treated for 20 min with pepsin at 37 °C, washed, incubated for 2 h with 50% formamide in 300 mM NaCl and 30 mM saline sodium citrate (pH 7.0) at 65 °C, and washed twice in saline sodium citrate buffer. The DNA was denatured for 30 min at 37 °C with 2 M HCl, washed, neutralized for 10 min with 0.1 M borate buffer (pH 8.5), washed, and blocked with 0.05% Tween 20 and 5% FCS in PBS (pH 7.4). The samples were then stained with 5 µg mL^{-1} BrdU-specific antibody (Roche, Mannheim, Germany) for 45 min at 22 °C, washed, and stained with Cy3-coupled $F(ab)_2$ anti-mouse IgG (Jackson ImmunoResearch). Serial sections were counted by a “blinded” investigator.

For doublecortin staining, the samples were demasked in citrate buffer after rehydration, incubated in a microwave for 15 min at 650 W, washed in PBS, blocked in blocking solution (Candor Biosciences, Wangen, Germany), stained with antibodies against doublecortin (1:100, Abcam, Cambridge, UK) for 45 min at 22 °C, washed 3 times in PBS/0.05% Tween 20, stained with Cy3-anti-rabbit $F(ab)_2$ fragments, washed again, and embedded in Mowiol.

Anti-BrdU and GFAP doublestainings: After staining with BrdU-specific antibodies, the samples were incubated with antibodies for GFAP (1:1000, Dako, Eching, Germany) for 45 min at 22 °C, washed 3 times in PBS/0.05% Tween 20 and once in PBS, stained with FITC anti-rabbit IgG $F(ab)_2$ fragments for 45 min at 22 °C, washed again, and embedded in Mowiol.

For BrdU and NeuN doublestainings, samples were processed as above for BrdU, incubated with BrdU-specific antibodies for 45 min at 22 °C, washed 3 times in PBS/0.05% Tween 20 and once in PBS, fixed for 15 min in 2% PFA/PBS (pH 7.3), washed 4 times with PBS, demasked in citrate buffer for 15 min in a microwave at 650 W, washed, blocked with PBS/0.05% Tween/5% FCS for 15 min, washed, incubated with antibodies against NeuN (1:100; Millipore, Schwalbach, Germany) for 45 min at 22 °C, washed 3 times in PBS/0.05% Tween 20 and once in PBS, stained with Cy3-anti-rat IgG $F(ab)_2$ fragments (1:100) and FITC-anti-mouse IgG $F(ab)_2$ fragments (1:500) for 45 min at 22 °C, washed, and embedded.

Neurogenesis and neuronal survival were determined by BrdU labeling 1 day or 21 to 28 days before the mice were sacrificed. Maturation was analyzed by doublecortin staining. Every tenth section of serial sections of the hippocampus was counted.

Western blots: The hippocampal area was removed, immediately shock frozen, and homogenized with a tip sonicator in 100 µl 0.1% SDS, 25 mM HEPES, 0.5% deoxycholate, 0.1% Triton X-100, 10 mM EDTA, 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 125 mM NaCl, and 10 µg g^{-1} aprotinin/leupeptin. Samples were lysed for 5 min at 4 °C and centrifuged at 14,000 rpm for 5 min at 4 °C, after which 20 µL 5x SDS-Laemmli buffer was added. Proteins were separated by 8.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), incubated with either an antibody specific for phospho-Ser-473-Akt or for Akt (both diluted 1:1,000, Cell Signalling, Frankfurt, Germany) for 1 h at 22 °C, washed, and developed with

alkaline phosphatase-coupled secondary antibodies with the Tropix chemoluminescence system.

Behavioral studies: Behavioral testing was performed between 3:00 P.M. and 6:00 P.M. If appropriate, animals were video tracked by a camera (Noldus-Systems, Worpswede, Germany). All tests were performed on separate days. Experiments were performed with diffuse indirect room light. **Novelty-suppressed feeding** was measured as the length of time during which the mice explored a new environment before they began eating after a fasting period of 24 h. For the **forced swim test**, mice were placed in a cylinder filled with water (21–23 °C) for 15 min. After 24 h the mice were again placed in a water-filled cylinder for 6 min, and the time of mobility during the last 4 min of the second trial was recorded. Mice were judged immobile when they moved only to keep their heads above water. A quadratic **open-field arena** was used with sides 50 cm long, a white plastic floor, and sidewalls 30 cm high. Each animal was released near the wall and observed for 30 min. The open field was defined as 10 cm away from the wall. The **light/dark box test** consisted of a dark and safe compartment and a brightly illuminated, open, and thus aversive area. An aperture of 5×5 cm with rounded-down corners led from the light area to the dark box. Each mouse was released in the dark compartment and observed for 5 min. **Coat state** was scored on the head, neck, back, and ventrum with either a zero for a normally groomed coat or a 1 for an unkempt coat at each site. The **elevated plus maze** was performed with a maze consisting of two side-shielded arms and two open arms (height, 40 cm). Mice were placed into the central area, and behaviour was tested for 5 min. In the **splash test**, 200 µL of a 10% sucrose solution was spotted onto the mouse's snout, and the latency to begin grooming and the grooming frequency over 5 min were measured. Control experiments showed that the different genotypes did not differ in their locomotion.

Egg-Laying Assay: *asm-1(kk-1)* worms and wild type *C. elegans* Bristol strain (N2) (from Caenorhabditis Genetics Center, University of Minnesota, Minneapolis, USA) were cultured at 20 °C and fed with Escherichia coli OP50 as a food source. Egg laying was counted from young adults that were picked as L4 larvae from OP50 plates and transferred to fresh bacteria plates before the assay. After development for 20 h at 20 °C, worms from these plates were then transferred individually to 96-well microplates containing 50 µL M9 buffer (20 mM KH_2PO_4, 40 mM Na_2HPO_4, 80 mM NaCl, 1 mM $MgSO_4$) per well with or without 1 mM fluoxetine or 12.5 mM 5-

hydroxytryptamine. The number of eggs released was scored after 90 min at room temperature. Each assay contained 6 to 8 worms for each experimental group.

Hippocampal ceramide injection and behavior: Adult male C57BL/6J mice (Charles River, Germany) were deeply anaesthetized with 1 g kg^{-1} ketamine and 76 mg kg^{-1} medetomidine hydrochloride i.p. and 100 mg kg^{-1} metamizol s.c. The animal was placed in a Kopf stereotaxic frame. Two injection cannulas (Microbiotech/se AB, Stockholm, Sweden) were aimed at the dorsal hippocampus (AP -2.0; ML ± 1.5; DV -2.3 mm) using coordinates relative to bregma, and fixed in place using two anchor screws (stainless steel, d=1.4 mm) and dental cement. Animals were kept warm and allowed to recover from anaesthesia. Animals were then returned to their home cage for single housing and monitored daily, allowing at least 4 days for complete recovery. All animals received seven bilateral injections with either 2 µM C16 ceramide (Cayman, Ann Arbor, USA) or ghost micelles. C16 ceramide solution was prepared in 2% octyl ß-D-glucopyranoside and sonicated for 10 min prior to use. The ceramide test dose was determined after a dose-response test (2–200 µM). In that, 2 µM ceramide had no acute adverse effects on behavior. All injections were spaced 48 h apart. The injection volume was 0.2 µL per side, delivered at a flow rate of 0.1 µL min^{-1}. After injection, cannulas were left in place for 1 min to allow for diffusion. After the 7^{th} injection, coat state was scored and one day later, novelty-suppressed feeding was tested as described above. Then animals received two more ceramide injections spaced 48 h apart before sucrose preference was tested on four consecutive days. Each animal received two bottles constantly available, one filled with a 2% sucrose solution and one with water. Percent preference of sucrose vs. water was measured every day. After the behavioral tests, animals were sacrificed by cervical dislocation, and brains were removed for verification of probe localization[37].

Statistical analysis: Data were examined with analysis of variance (ANOVA) and post hoc tests. A *P* value of 0.05 or less (two-tailed) was considered indicative of statistical significance.

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FIGURE LEGENDS

Fig. 1: Amitriptyline and fluoxetine reduce hippocampal ceramide concentrations by inhibiting acid sphingomyelinase activity

(a) Acid sphingomyelinase (human: ASM) activity in human H4 neural cells at different concentrations of amitriptyline or fluoxetine. Therapeutic plasma concentrations of the antidepressants are indicated. Means ± s.d. of three independent experiments. Absolute values of ASM activity at 0.1 µM amitriptyline or fluoxetine were 6.08 ± 2.17 or 6.67 ± 3.07 nmol mg protein^{-1} h^{-1}, respectively. (**b**) Acid sphinfomyelinase (mouse: Asm) activity in the hippocampus of WT and Asm-transgenic (t-Asm) mice is dose-dependently inhibited by amitriptyline (Ami.) and fluoxetine (Fluox.). Means ± s.d. from WT mice (n=6–12 per group) and t-Asm mice (n=6–8 per group). Doses of amitriptyline and fluoxetine in mg L^{-1}. *, $P < 0.05$ compared to untreated WT mice; $^{\Delta}$, $P < 0.05$ compared to untreated t-Asm mice, ANOVA. (**c**) Immunostainings of hippocampal sections using Cy3-coupled antibodies against Asm show reduced expression of Asm in the dentate gyrus (upper row) and CA3 region (lower row) of amitriptyline or fluoxetine treated compared to untreated mice. Representative results from 9–12 mice per group; magnification 200×. **(d)** Ceramide levels in unstressed or corticosterone (corticost.)-stressed mice that were treated with amitriptyline and fluoxetine or left untreated. Doses of amitriptyline and fluoxetine in mg L^{-1}. Means ± s.d. (180 mg L^{-1} amitripytline and 80 mg L^{-1} fluoxetine: WT, Asm-deficient: n=12, t-Asm: n=9, all other doses: n=5; Ac-heterozygous mice: n=4.). *, $P < 0.05$ compared to untreated WT mice; $^{\Delta}$, $P < 0.05$ compared to the corresponding untreated mice; ANOVA. (**e**) Immunostainings of hippocampal sections with Cy3-coupled antibodies against ceramide show reduction of ceramide in the dentate gyrus (upper row) and CA3 regions (lower row) after treatment of WT mice with amitriptyline or fluoxetine. Representative figures from 12 mice each.

Fig. 2: The acid sphingomyelinase/ceramide system is required for the neurobiological effects of antidepressants

Neurogenesis (**a**), neuronal maturation (**b**), and neuronal survival (**c**) in the hippocampus of unstressed or corticosterone-stressed mice that were treated with amitriptyline or fluoxetine. Asm-deficient mice did not respond to the antidepressants

(**a–c**). Asm-deficient mice exhibit a constitutive increase in neurogenesis, neuronal maturation, and neuronal survival rates, while these parameters are decreased in t-Asm and Ac-heterozygous mice (**a–c**). Mean ± s.d. (n=12 WT, t-Asm, and Asm-deficient mice in **a** and n=6 each in **b**, **c**; n=5 Ac-heterozygous mice). *, $P < 0.05$ compared to untreated WT mice; $^{\Delta}$, $P < 0.05$ compared to corresponding mice, ANOVA). (**d**) Western blots showing constitutive and antidepressants-induced phosphorylation of Ser 473 of Akt. Aliquots of the samples were blotted with antibodies against Akt (lower blots). The blots are representative of results from 4–6 mice/group. (**e**) Fluoxetine- and 5-Hydroxytryptamine (5-HT) induced egg laying in WT, *asm*-1 and *mod-5* deficient *C. elegans*. Mean ± s.d. from 6–8 worms per group. *, $P < 0.05$ compared to untreated WT worms; $^{\Delta}$, $P < 0.05$ compared to untreated corresponding genotype; ANOVA. (**f,g**) Asm activity neurogenesis and neuronal survival in non-stressed and stressed mice treated with fendiline or left untreated. Mean ± s.d. from each 6 (**f** and neurogenesis) or 4 (survival) mice. *, $P < 0.05$ compared to untreated WT mice; $^{\Delta}$, $P < 0.05$ compared to untreated Asm-deficient mice; ANOVA.

Fig. 3: Behavioral effects of antidepressant drugs can be mediated by the inhibition of acid sphingomyelinase

Novelty-suppressed feeding (**a**), coat (**b**), splash (**c**), open-field (**d**), light/dark box (**e**), and forced swim (**f**) tests in non-stressed and corticosterone-stressed mice treated with antidepressants, fendiline or left untreated. Mean ± s.d., number of mice per group in **a** from left to right: 41, 42, 19, 9, 12, 12, 12, 9, 33, 28, 14, 9, 12, 12, 12, 8, 19, 14, 12, 10, 12, 12, 12, 10, otherwise WT, Asm-deficient, t-Asm mice: n=6, Ac-heterozygous mice: n=5. *, $P < 0.05$ compared to untreated WT mice; $^{\Delta}$, $P < 0.05$ compared to untreated control of each genotype, ANOVA.

Fig. 4: Hippocampal ceramide has a critical role in depression-like phenotypes

Ceramide levels (**a**) and neurogenesis (**b**) in unstressed or corticosterone-stressed mice treated with PDMP and/or amitriptyline or fluoxetine. Heterozygosity of the Asm in Ac-heterozygous mice reduces ceramide levels and improves neurogenesis compared to Ac-heterozygous mice. Doses of amitriptyline and fluoxetine in mg L^{-1}. Mean ± s.d. from 5 mice each. *, $P < 0.05$ compared to untreated WT mice; $^{\Delta}$, $P < 0.05$ compared to PDMP alone or corticosterone + PDMP as appropriate; $^{\#}$, $P < 0.05$

compared to corticosterone alone as appropriate; ANOVA. (**c**) Injection of C16 ceramide (Cer) into the hippocampus results in depression-like symptoms as assessed by reduced sucrose preference and prolonged latency to feed. Mean ± s.e.m. from 5 mice for vehicle treated control (Veh) and 8 mice for Cer *, $P < 0.05$, t-test. (**d,e**) Hippocampal ceramide, neurogenesis and maturation in WT and Asm-deficient mice subjected to chronic unpredictable stress (CUS) and treated with amitriptyline or fluoxetine or left untreated. Mean ± s.d. from 5 mice each. *, $P < 0.05$ compared to untreated WT mice; $^{\Delta}$, $P < 0.05$ compared to CUS-treated mice of each genotype, ANOVA.

Fig. 1

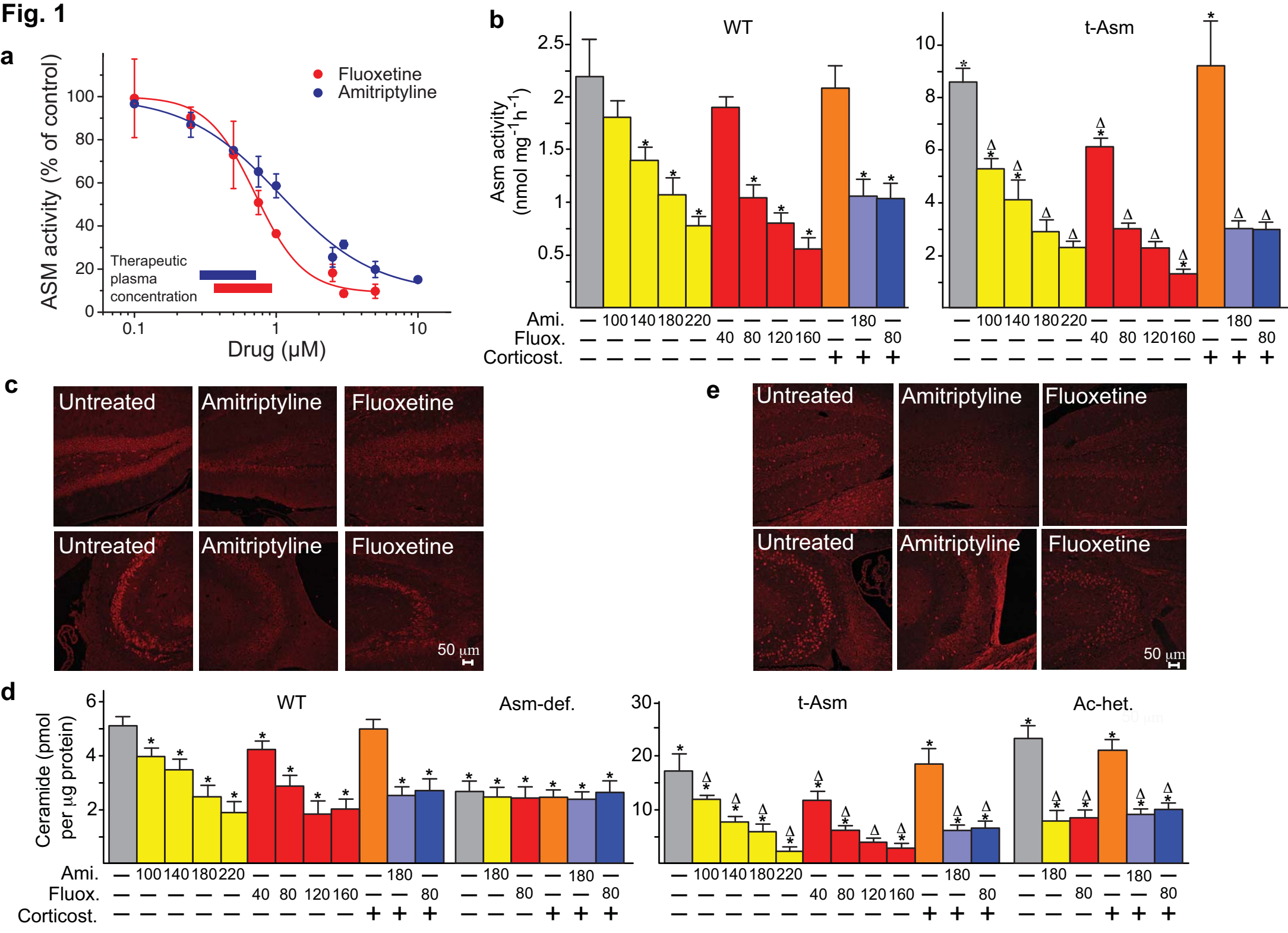

a
ASM activity (% of control)
Fluoxetine
Amitriptyline
Therapeutic plasma concentration
Drug (μM)
b
WT
t-Asm
Asm activity (nmol mg-1h-1)
Ami.
Fluox.
Corticost.
c
Untreated
Amitriptyline
Fluoxetine
50 μm
e
Untreated
Amitriptyline
Fluoxetine
50 μm
d
WT
Asm-def.
t-Asm
Ac-het.
Ceramide (pmol per μg protein)
Ami.
Fluox.
Corticost.

Fig. 2

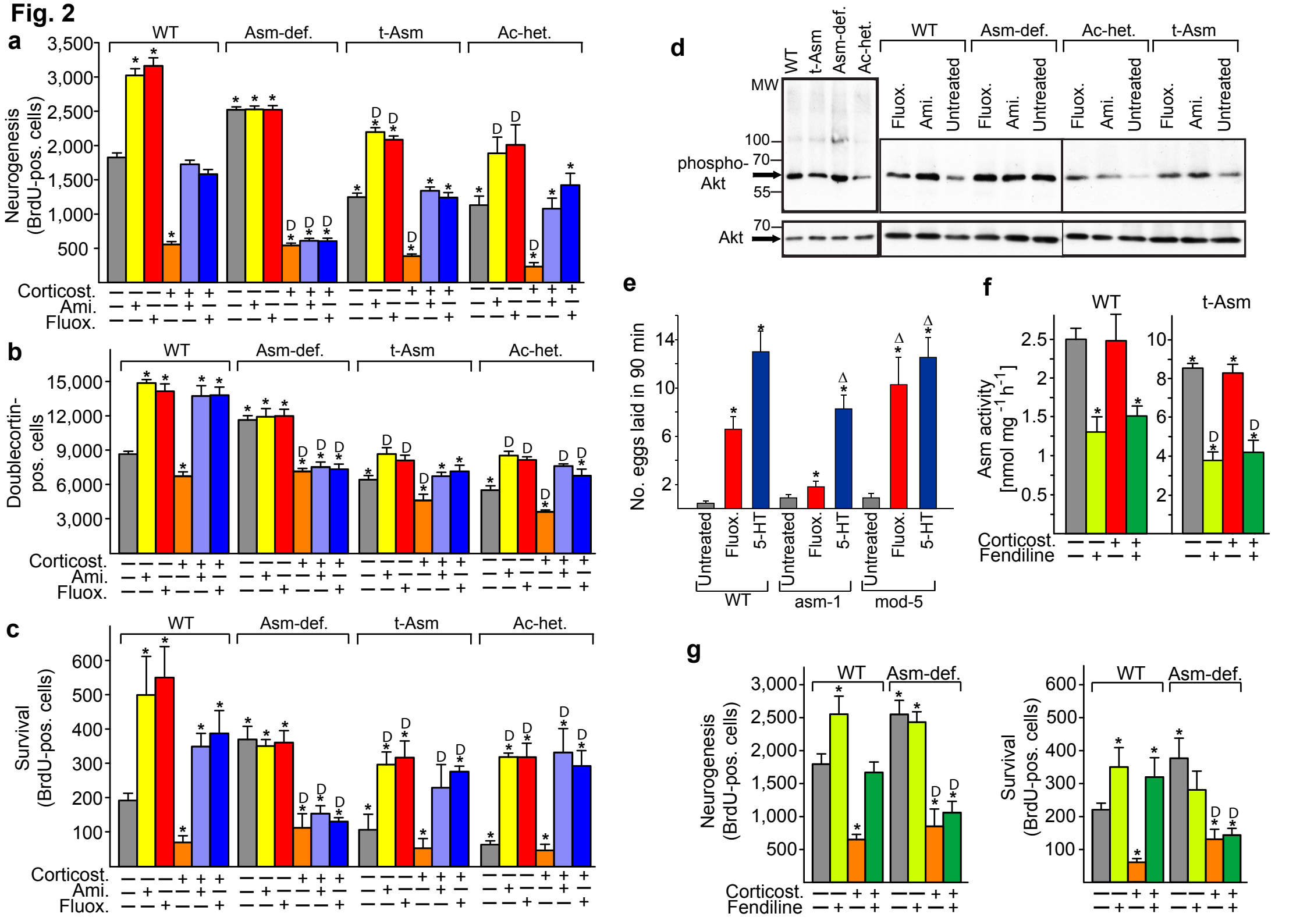

a
WT
Asm-def.
t-Asm
Ac-het.
Neurogenesis (BrdU-pos. cells)
3,500
3,000
2,500
2,000
1,500
1,000
500
Corticost.
Ami.
Fluox.
b
Doublecortin-pos. cells
15,000
12,000
9,000
6,000
3,000
c
Survival (BrdU-pos. cells)
600
500
400
300
200
100
d
MW
WT
t-Asm
Asm-def.
Ac-het.
Fluox.
Ami.
Untreated
100
70
55
phospho-Akt
Akt
e
No. eggs laid in 90 min
14
10
6
2
Untreated
Fluox.
5-HT
WT
asm-1
mod-5
f
Asm activity [nmol mg -1 h-1]
2.5
2
1.5
1
0.5
10
8
6
4
2
Corticost.
Fendiline
g
Neurogenesis (BrdU-pos. cells)
3,000
2,500
2,000
1,500
1,000
500
Survival (BrdU-pos. cells)
600
500
400
300
200
100

Fig. 3

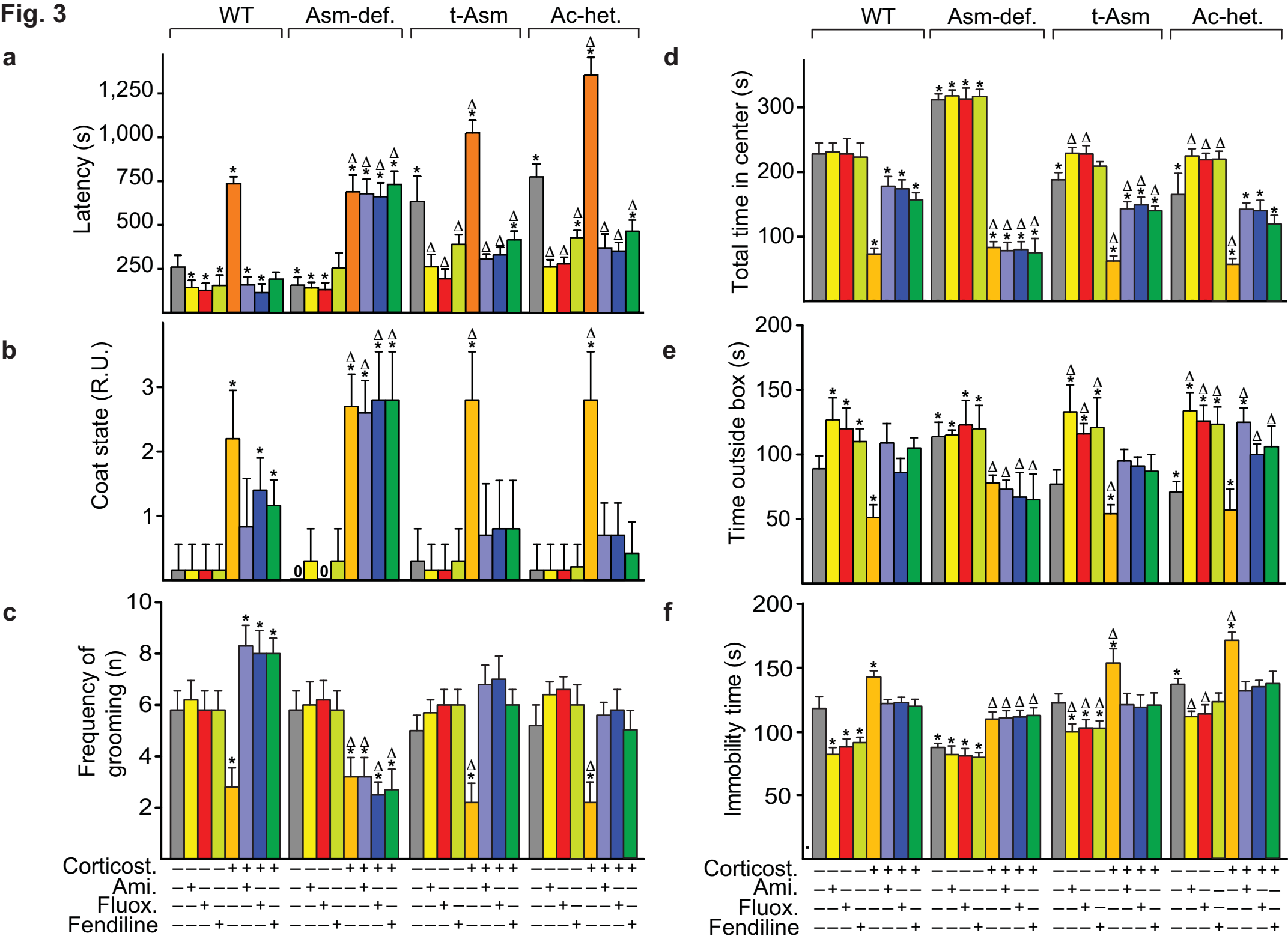
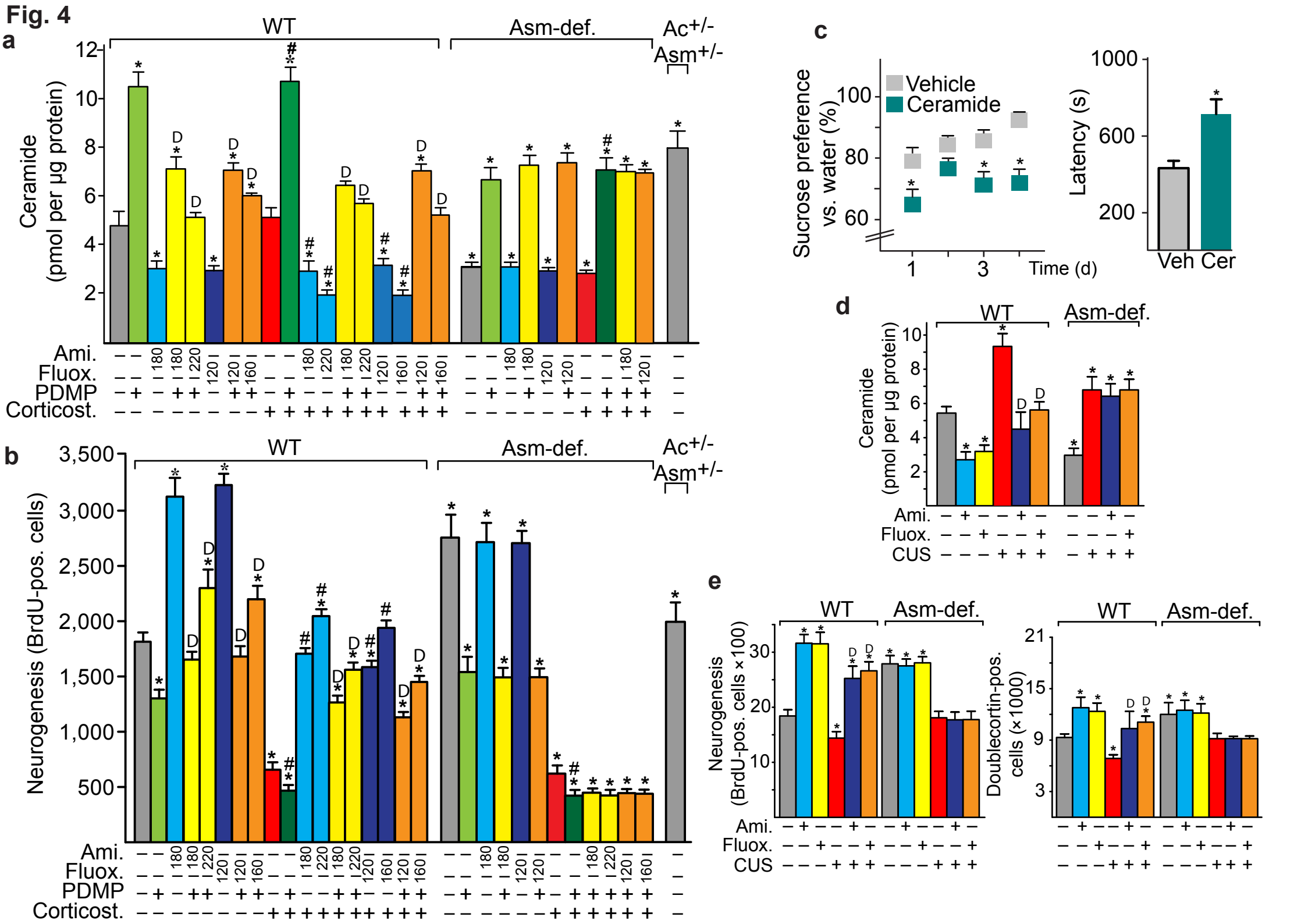

WT
Asm-def.
t-Asm
Ac-het.
a
Latency (s)
1,250
1,000
750
500
250
b
Coat state (R.U.)
3
2
1
0
0
c
Frequency of grooming (n)
10
8
6
4
2
d
Total time in center (s)
300
200
100
e
Time outside box (s)
200
150
100
50
f
Immobility time (s)
200
150
100
50
Corticost. – – – + + + + – – – + + + + – – – + + + + – – – + + + +
Ami. – + – – – + – – – + – – – + – – – + – – – + – – – +
Fluox. – – + – – – + – – + – – – + – – + – – – + – – – +
Fendiline – – – + – – – + – – – + – – – + – – – + – – – + – – – +

Fig. 4


a
WT
Asm-def.
Ac+/-
Asm+/-
Ceramide (pmol per µg protein)
Ami.
Fluox.
PDMP
Corticost.
b
Neurogenesis (BrdU-pos. cells)
c
Vehicle
Ceramide
Sucrose preference vs. water (%)
Time (d)
Latency (s)
Veh
Cer
d
CUS
e
Doublecortin-pos. cells (×1000)