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# Selective Knockdown of TASK3 Potassium Channel in Monoamine Neurons: A New Therapeutic Approach for Depression

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Running title: Intranasal delivery of conjugated TASK3-siRNA

## Abstract

Current pharmacological treatments for major depressive disorder (MDD) are severely compromised by both slow action and limited efficacy. RNAi strategies have been used to evoke antidepressant-like effects faster than classical drugs. Using small interfering RNA (siRNA), we herein show that TASK3 potassium channel knockdown in monoamine neurons induces antidepressant-like responses in mice. TASK3-siRNAs were conjugated to cell-specific ligands, sertraline (Ser) or reboxetine (Reb), to promote their selective accumulation in serotonin (5-HT) and norepinephrine (NE) neurons, respectively, after intranasal delivery. Following neuronal internalization of conjugated TASK3-siRNAs, reduced TASK3 mRNA and protein levels were found in the brainstem 5-HT and NE cell groups. Moreover, Ser-TASK3-siRNA induced robust antidepressant-like behaviors, enhanced the hippocampal plasticity and potentiated the fluoxetine-induced increase on extracellular 5-HT. Similar responses, yet of lower magnitude, were detected for Reb-TASK3-siRNA. These findings provide substantial support for TASK3 as a potential target, and RNAi-based strategies as a novel therapeutic approach to treat MDD.

**Keywords:** RNAi, depression, new antidepressant target, $K_{2P}$ channel, intranasal delivery

## Introduction

The high prevalence and increasing socioeconomic impact of major depressive disorder (MDD) is not paralleled by improvements in its treatments **[1-3]**. The antidepressant drugs available for MDD treatment, including the widely prescribed monoamine (serotonin -5-HT- and norepinephrine –NE-) reuptake inhibitors, are suboptimal and still unsatisfactory. Current antidepressant drugs have a slow onset of action and limited efficacy, which results in a high percentage of MDD patients with poor or no therapeutic responses, thus reducing quality of life and increasing suicide risk **[4-6]**. The reasons for the limited efficacy of monoamine-based treatments are manifold, including neurobiological adaptive mechanisms and genetic factors, resulting in a great individual variability. Understanding the neurobiological basis of MDD and improving the therapeutic strategies currently available remains one of the foremost challenges for modern neuropsychopharmacology.

RNAi strategies may avoid some of the stated limitations once adequate administration routes to CNS and targeting of selected neuronal populations are established **[7, 8]**. Previous studies showed that the intracerebroventricular (i.c.v.) or intranasal (i.n.) administration of selective serotonin reuptake inhibitor (SSRI)-conjugated siRNA (small interfering RNA) targeting the serotonin transporter (SERT) or 5-$HT_{1A}$-autoreceptors induced robust antidepressant-like responses in mice **[9, 10]**. These effects were similar to those induced by the local application of unmodified siRNA sequence in the raphe nuclei **[11, 12]**. The presence of sertraline (Ser) in the conjugated siRNA allows its selective accumulation in 5-HT neurons and the targeting of genes expressed by these neurons. Hence, 1-week i.n. administration of small amounts (2.1 nmol/day) of a Ser-conjugated SERT-siRNA evoked antidepressant-like responses comparable to those induced by 1-month treatment with fluoxetine 10 mg/kg/day, i.p. **[10]**. Likewise, acute 5-$HT_{1A}$-autoreceptor knockdown also evoked rapid antidepressant-like effects in mice, due to a lesser self-inhibition of serotonergic neurons, thus increasing stress resilience **[9, 11]**.

Here we extend this strategy to knockdown TASK3, an acid-sensitive two-pore domain potassium channel ($K_{2P}$) (referred as TWIK-related acid sensitive potassium channel 3, also known as $K_{2P}$9.1) **[13]**. TASK3 and other related potassium channel members have been implicated in the pathophysiology of MDD and in the antidepressant drug respose as well as in resilience mechanisms.

In turn, TASK3 channels may have therapeutic potential in neuropsychiatric disorders due to their ability to control the resting membrane potential and neuronal excitability **[14-16]**. Preclinical studies showed that full deletion of the TASK3 channel in mice markedly reduced REM sleep and evoked antidepressant-like effects **[17]**. Likewise, non-selective TASK3 antagonist administration increased the active alertness with a concurrent decrease in both REM and delta sleep in wild-type mice, suggesting their therapeutic antidepressant potential **[18]**. Human and rodent TASK3 channels are abundantly expressed in the cerebral cortex, hippocampus (HPC), thalamic and hypothalamic nuclei, and cerebellum as well as in 5-HT and NE neurons of the dorsal raphe nucleus (DR) and *locus coeruleus* (LC), respectively **[19-23]**.

Taking advantage of the neuronal selectivity of conjugated siRNAs, we examined the potential antidepressant effects of TASK3 knockdown in 5-HT and NE neurons under the working hypothesis that the reduced TASK3 expression would increase the neuronal excitability and therefore, facilitate the monoaminergic neurotransmission in a similar way to that produced by 5-$HT_{1A}$-autoreceptor knockdown **[9, 11]**. Further, the selective reduction of TASK3 expression in 5-HT or NE neurons would avoid potential side effects of TASK3 channel blockers derived from the interaction with cortical, hippocampal and cerebellar TASK3 channels **[24]**.

## Materials and Methods

### Animals

Male C57BL/6J mice (10–14 weeks; Charles River, Lyon, France) were housed under controlled conditions (22±1ºC; 12h light/dark cycle) with food and water available *ad-libitum*. Animal procedures were conducted in accordance with National (Royal Decree 53/2013) and European legislation (Directive 2010/63/EU on the protection of animals used for scientific purposes, 22 September 2010), and were approved by the Institutional Animal Care and Use Committee of the University of Barcelona.

### siRNA Synthesis

Synthesis and purification of naked and conjugated siRNA targeting TASK3 channel (TASK3-siRNA, nt: 1056–1075, GenBank accession NM_001033876) and nonsense siRNA (NS-siRNA) were performed by nLife therapeutics S.L. (Granada, Spain). Both TASK3-siRNA and NS-siRNA sequences were conjugated with the SSRI sertraline (Ser-TASK3-siRNA and Ser-NS-siRNA) as described to target selectively 5-HT neurons **[9, 10]**. Moreover, TASK3- and NS-siRNA sequences were also conjugated with the selective NE reuptake inhibitor reboxetine (Reb-TASK3-siRNA and Reb-NS-siRNA) to reach NE neurons. Details are shown in the Supplementary Information.

To confirm the *in vivo* incorporation of conjugated siRNA into 5-HT or NE neurons, Ser-NS-siRNA and Reb-NS-siRNA were additionally labeled with Alexa488 in the antisense strand (A488-Ser-NS-siRNA or A488-Reb-NS-siRNA). Control groups received Alexa488-PBS (A488-PBS) or non-conjugated (naked) NS-siRNA (A488-NS-siRNA) using an identical procedure to Ferrés-Coy et al. and Alarcón-Arís et al. **[10, 25]**. Stock solutions of all siRNAs were prepared in RNAse-free water and stored at -20ºC until use. Sequences are shown in **Suppl. Table 1**.

### Treatments

Mice received 1) unmodified siRNAs locally infused into dorsal raphe nucleous (DR) or *locus coeruleus* (LC), or 2) conjugated siRNAs administered intranasally (i.n). For the intracerebral infusion

of TASK3-siRNA and NS-siRNA, mice were anesthetized (pentobarbital, 40 mg/kg, i.p.) and silica capillary microcannulae (110μm-OD and 40μm-ID; Polymicro Technologies, Madrid, Spain) were stereotaxically implanted into DR (coordinates in mm: AP, -4.5; ML, -1.0; DV, -4.1; with a lateral angle of 20º) or LC (AP, -5.2; ML, -0.9; DV, -3.5) **[26]**. Local siRNA microinfusion was performed 24h after surgery in awake mice using a precision minipump at a 0.5 μl/min as previously described **[11, 12]**. siRNAs were prepared in a RNase-free artificial cerebrospinal fluid (aCSF) with 5% glucose and infused intra-DR or intra-LC once daily at the dose of 10 μg/μl (0.7 nmol/dose). Mice received two doses in 2 consecutive days. Control mice received aCSF. Mice were sacrificed 24h after last infusion.

For i.n. administration, mice were slightly anesthetized with 2% isoflurane inhalation and placed in a supine position **[9, 10, 25]**. A 5 μl-drop of phosphate buffered saline (PBS) or conjugated siRNAs (Ser- or Reb-NS-siRNA or Ser- or Reb-TASK3-siRNA) was applied alternatively to each nostril once daily. A total of 10 μl of solution containing 30 or 75 μg (2.1 or 5.3 nmol/day) of conjugated siRNA was delivered for 7 days and mice were sacrificed between 3-4 days after last administration.

***In situ* Hybridization**

*In situ* hybridization was performed as previously described **[9, 10]**. Antisense oligoprobes (Göttingen, Germany) were complementary to the following bases: TASK3/839-888 (GenBank accession NM_001033876), TASK1/101-150 (NM_010608), TREK1/592-641 (NM_001159850), SERT/820-863 (NM_010484.1), serotonin-1A receptor-5-$HT_{1A}$R/1780-1827 (NM_008308), NET/1210-1259 (NM_009209), $\alpha_2$-adrenoreceptor-Adra2/2137-2186 (NM_NC_000085), brain derived neurotrophic factor-BDNF/1188-1238 (NM_007540), vascular endothelial growth factor-VEGF/2217-2267 (NM_001025250) and activity regulated cytoskeletal protein-ARC/1990-2040 (NM_018790). Details are shown in Supplementary Information.

**Immunohistochemistry**

Mice were anesthetized with pentobarbital and transcardially perfused with 4% paraformaldehyde in sodium-phosphate buffer (pH 7.4). Brains were collected, post-fixed 24h at 4ºC in the same solution, and then placed in gradient sucrose solution 10–30% for 3 days at 4ºC. After cryopreservation, serial 30 µm-thick sections were cut through hippocampal formation (HPC), midbrain raphe nuclei and LC. Immunohistochemical procedure was performed for doublecortin (DCX), glial fibrillary acidic protein (GFAP), Iba-1, Ki-67, tyrosine hydroxylase (TH) and tryptophan hydroxylase 2 ($TPH_2$) using biotin-labeled antibody procedure **[9, 10]**. Details are shown in the Supplementary Information.

**Confocal Fluorescence Microscopy**

Intracellular Ser- and Reb-NS-siRNA distribution in 5-HT and NE neurons was examined using a Leica TCS SP5 laser scanning confocal microscope (Leica Microsystems Heidelberg GmbH, Manheim, Germany) equipped with a DMI6000 inverted microscope, blue diode (405nm), Argon (458/476/488/496/514), diode pumped solid state (561nm) and HeNe (594/633nm) lasers. Details are shown in the Supplementary Information.

**Western Blot Analysis**

Mice were sacrificed with pentobarbital overdose, brains were rapidly removed, and medial prefrontal cortex (mPFC), HPC, DR and LC were dissected and snap-frozen on dry ice. Tissues were homogenized in RIPA buffer with protease inhibitors and total protein amount was quantified using a bicinchoninic acid (BCA) kit (Thermo Scientific Scientific Inc, IL, USA). Details are shown in the Supplementary Information.

**Drugs**

All reagents used were of analytical grade and were obtained from Merck (Germany). 5-HT oxalate, NE bitartrate, (±)-8-hydroxi-2(dipropylamino)tetralin hydrobromide (8-OH-DPAT), and clonidine were from Sigma-Aldrich-RBI (Madrid, Spain). Fluoxetine, reboxetine, citalopram hydrobromide and desipramine were from Tocris (Madrid, Spain). To assess the local effects in microdialysis procedures, drugs were dissolved in artificial cerebrospinal fluid (aCSF) and were administered by reverse dialysis

at the stated concentrations **[9, 10]**. All other drugs dissolved in saline or aCSF as required. Concentrated solutions (1mM; pH adjusted to 6.5–7 with $NaHCO_3$ when necessary) were stored at -80ºC and working solutions were prepared daily by dilution in aCSF.

**Intracerebral Microdialysis**

Extracellular 5-HT and NE concentrations were evaluated by *in vivo* microdialysis as previously described **[9, 10, 27-29]**. A full description is given in Supplementary Information.

**Behavioral Studies**

Behavioral analyses were performed 24h after the last dose with an interval of one day between tests. All mice were evaluated in two behavioral paradigms including: 1) novelty suppressed-feeding test (NSFT) and 2) tail suspension test (TST) or forced swim test (FST) or marble burying test (MBT). All tests were performed between 10:00-15:00h by an experimenter blind to mouse treatments. On the test day, mice were placed in a dimly illuminated behavioral room and were left undisturbed for at least 1h before testing. In an additional group, the extracellular 5-HT concentration in mPFC was simultaneously measured during TST paradigm by *in vivo* microdialysis **[11]**. Details are shown in the Supplementary Information.

**Statistical Analyses**

Results are given as mean ± S.E.M. Data were analyzed using GraphPad Prism 7.01 (San Diego, CA). Statistical analyses were performed by two-tailed Student's *t*-test and one-way or two-way ANOVA followed by Tukey's *post-hoc* test as appropriate. In NSFT, we used the Kaplan-Meier survival analysis following Mantel-Cox log-rank **[30]**. Animals that did not eat during the 10 min testing time were discarded from the analysis. Differences were considered significant when $p < 0.05$.

## Results

### Selective Intranasal Delivery of Sertraline- and Reboxetine-conjugated TASK3-siRNA Downregulates TASK3 Expression in 5-HT and NE Neurons

First, we examined whether a non-conjugated siRNA sequence targeting TASK3 (TASK3-siRNA) could reduce *in vivo* TASK3 channel expression in the DR and LC. Mice were injected intra-DR or unilaterally intra-LC with 1 µl of: 1) vehicle, 2) nonsense siRNA (NS-siRNA) or 3) TASK3-siRNA (0.7 nmol/dose) for two consecutive days. *In situ* hybridization experiments revealed reductions of TASK3 mRNA levels to 69 ± 4% ($p=0.0002$, DR) and 82 ± 2% ($p<0.0001$, LC) compared to control values (vehicle and NS-siRNA) (**Suppl. Fig. 1**).

Next, conjugated siRNA molecules were synthesized by the manufacturer using a previously developed strategy, in which the SSRI sertraline was covalently bound to the siRNA (Ser-siRNA) in order to selectively target 5-HT neurons after i.n. or i.c.v. administration **[9, 10]**. Using the same procedure, siRNA sequences were also conjugated with the selective norepinephrine transporter (NET) inhibitor reboxetine (Reb-siRNA) to promote their accumulation in NE neurons. To assess the distribution of conjugated siRNA in the DR and LC neurons after i.n. administration, mice were treated once daily for 4 days with: 1) A488-PBS, 2) A488-NS-siRNA, 3) A488-Ser-NS-siRNA or 4) A488-Reb-NS-siRNA (2.1 nmol/dose). Mice were killed 6 h after the last dose. Confocal fluorescence microscopy revealed the presence of double-conjugated molecules in DR and LC. Hence, A488-Ser-NS-siRNA was intracellularly detected in $TPH_2$-positive 5-HT neurons of DR –but not in TH-positive neurons of LC– **(Fig. 1a)** and, A488-Reb-NS-siRNA was found in TH-positive NE neurons of LC – but not in $TPH_2$-positive neurons of DR– **(Fig. 1b)** indicating the selective incorporation of the oligonucleotides in each monoamine neuronal group expressing the corresponding transporters. Although the traffic mechanisms occurring after the internalization of conjugated siRNA molecules in monoamine neurons are not fully understood, endosomal networks would be involved, since Ser- or Reb-conjugated A488-siRNAs co-localized with the late endosomal marker Rab7 in DR and LC (**Fig. 1c**). Like antisense oligonucleotide molecules (ASO) conjugated with indatraline (triple monoamine reuptake inhibitor) **[25]**, the conjugated siRNAs used here were scarcely detected in the olfactory

bulbs, much closer to the application site than the brainstem monoamine nuclei. Remarkably, A488-Reb-NS-siRNA molecules were not found in TH-positive dopamine (DA) neurons of the olfactory bulb expressing the DA but not the NE transporter **(Suppl. Fig. 2)**.

Next step was to examine the effect of Ser-TASK3-siRNA on DR TASK3 mRNA expression. Ser-TASK3-siRNA (2.1 nmol/day) was administered i.n. for 7 days and two control groups were used treated with PBS or Ser-NS-siRNA. *In situ* hybridization experiments revealed a significant reduction of DR TASK3 mRNA level to 89 ± 2% of control groups ($p<0.001$), without affecting TASK3 expression in LC ($p=0.4656$) nor the expression of other genes expressed in 5-HT neurons such as 5-$HT_{1A}$ receptor ($p=0.0922$), SERT ($p=0.4021$), and other $K_{2P}$ channels (TASK1, $p=0.944$, TREK1, $p=0.1777$) **(Suppl. Fig. 3)**.

Given the relatively small reduction of DR TASK3 mRNA level, further experiments were performed with a higher daily dose (5.25 nmol/day) also administered during 7 days. TASK3 mRNA density was reduced to 83 ± 2% of PBS group ($p<0.05$) levels in the DR **(Fig. 2b)**, without altering TASK3 expression in the hippocampal formation (CA1 and dentate gyrus - DG) or medial prefrontal cortex (mPFC) **(Fig. 2c)**. A more exhaustive histological analysis revealed a greater effect of Ser-TASK3-siRNA on TASK3 mRNA expression. Dipped TASK3 hybridized sections immunostained by using a specific 5-HT neuronal marker ($TPH_2$) showed a reduced number of $TPH_2$-positive cells expressing TASK3 mRNA (60 ± 10% of PBS group) as well as a decreased intracellular TASK3 density (56 ± 6% of PBS group) in TASK3 knockdown mice along the DR anteroposterior axis **(Figs. 2a, 2d, 2e)**. Two-way ANOVA showed an effect of group $F(1,14)=14.72$, $p=0.0018$ and anteroposterior axis $F(1,14)=4.418$, $p=0.00541$ for double $TPH_2$- and TASK3-positive cells and, an effect of group $F(1,14)=17.86$, $p=0.0008$ for intracellular TASK3 mRNA density in $TPH_2$-positive neurons. Western blot analysis confirmed the selective silencing at the TASK3 protein level in DR ($p=0.04$) **(Figs. 2f, 2g)**.

Similarly, i.n. treatment with Reb-TASK3-siRNA (5.25 nmol/day for 7 days) reduced selectively TASK3 mRNA expression in the LC ($p=0.0181$) **(Fig. 3b)**, without affecting TASK3 mRNA level in other brain areas such as DR ($p=0.1625$), CA1, DG and mPFC ($p=0.347$) nor the expression of NET ($p=0.4063$), $\alpha_2$-adrenoreceptors ($p=0.8741$) and, TASK1 ($p=0.4023$) and TREK1

(p=0.9013) potassium channels in LC **(Fig. 3c, Suppl. Fig. 4)**. Co-localization analysis showed that Reb-TASK3-siRNA significantly reduced the number of TH-positive cells expressing TASK3 mRNA as well as the intracellular density of TASK3 mRNA (76 ± 5% of PBS group) in TASK3 knockdown mice **(Figs. 3a, 3d, 3e)**. Two-way ANOVA showed an effect of group for: 1) double TH- and TASK3-positive cells [(F(1,17)=17.98, p=0.0006] and 2) density in $TPH_2$-positive neurons [F(1,16)=30.44, p<0.0001], but not of anteroposterior axis nor interaction. Likewise, a reduction of TASK3 protein level was found in LC, but not in projection brain areas as mPFC and HPC (p=0.0236) **(Figs. 3f, 3 g)**.

Neither treatment with Ser-TASK3-siRNA nor Reb-TASK3-siRNA induced neuronal loss, as evidenced by the presence of the same number of $TPH_2$- or TH-positive neurons in all experimental groups (p=0.2787 and 0.3266, respectively) and the absence of immune responses such as astrogliosis (GFAP) or microglial activaction (Iba-1) **(Suppl. Fig. 5)**.

**Seven-day Treatment with Ser-TASK3-siRNA Evokes Neurochemical, Behavioral and Cellular Responses, Predictive of Clinical Antidepressant Activity**

First, to evaluate the neurochemical impact of i.n. treatment with Ser-TASK3-siRNA (5.25 nmol/day), we examined the effect on extracellular 5-HT concentration in mPFC (one of the projection areas of both DR and LC) using intracerebral microdialysis. There were no significant differences in baseline 5-HT concentration nor on veratridine-stimulated 5-HT values (**Suppl. Table 2**). However, the reduction of TASK3 expression in 5-HT neurons markedly attenuated the $5\text{-}HT_{1A}$-autoreceptor-mediated decline in 5-HT release, as shown by the dampened effect of 8-OH-DPAT (1 mg/kg, i.p.) on terminal 5-HT release **(Fig. 4a)**. Two-way ANOVA showed an effect of group F(1,17)=13.40, p=0.0019; time F(11,197)=7.692, p<0.0001 and interaction group-by-time F(11,187)=3.44, p=0.0002. In agreement with lesser $5\text{-}HT_{1A}$-autoreceptor-mediated self-inhibition of 5-HT neurons, Ser-TASK3-siRNA treatment augmented the effect of fluoxetine (20 mg/kg, i.p.) on extracellular 5-HT in mPFC **(Fig. 4b)**. Two-way ANOVA showed an effect of group F(1,10)=10.90, p=0.008; time F(15,150)=6.965, p<0.0001 and interaction group-by-time F(15,150)=2.104, p=0.0125.

Next, we evaluated the putative antidepressant-like effects of Ser-TASK3-siRNA by using behavioral paradigms. Seven-day Ser-TASK3-siRNA i.n. treatment significantly reduced the

immobility time in the tail suspension test (TST, p=0.0295), forced swim test (FST, p=0.0458) and shortened the latency to eat in the novelty suppressed feeding test (NSFT, Kaplan-Meier analysis showed p=0.0032) compared to PBS-treated mice **(Figs. 4c, 4d, 4e)**. In contrast, Ser-TASK3-siRNA administration did not affect obsessive/anxiety behavior assessed by the marble burying test (MBT, p=4834) **(Fig. 4f)**. Moreover, both experimental groups exhibited a similar locomotor activity in the open field test (OFT, data not shown).

Hippocampal neurogenesis and new synapses formation have been associated with clinical antidepressant actions **[31, 32]**. In agreement with this view, i.n. Ser-TASK3-siRNA treatment (7 days, 5.25 nmol/day) increased the number of Ki-67- (p=0.0389) **(Fig. 4g)** and DCX-positive (p=0.0388) cells in DG, compared to control group **(Fig. 4h)**. The enhanced proliferative and neurogenic activity induced by Ser-TASK3-siRNA were accompanied by a higher expression of neuroplasticity-associated genes, such as BDNF, ARC, and VEGF in different hippocampal subfields **(Figs. 4i, 4j)**. Two-way ANOVA showed an effect of group $F(1,40)=55.48$, $p<0.0001$; hippocampal subfields $F(3,40)=4.019$, $p<0.01$ and interaction group-by-subfields $F(3,40)=4.022$, $p=0.01$ for BDNF expression and, an effect of group $F(1,32)=44.15$, $p<0.0001$ and $F(1,36)=30.35$, $p<0.0001$ for ARC and VEGF expression, respectively.

**TASK3 Knockdown in Serotonergic Neurons Enhances 5-HT Release under Stressful Conditions**

Taking into account that 1) DR 5-$HT_{1A}$-autoreceptor **[9, 11]** and TASK3 knockdown mice evoked antidepressant-like effects, and 2) the existence of a functional interplay between both inhibitory mechanisms as evidenced by the dampened effect of 8-OH-DPAT in TASK3-treated mice, we examined whether the antidepressant-like effect of TASK3 knockdown was associated to an enhanced cortical 5-HT release during TST performance. No significant differences were observed in baseline 5-HT levels between Ser-TASK3-siRNA- and PBS-treated mice **(Suppl. Table 2)**. However, 5-HT release in TASK3 knockdown mice increased to ~240% of baseline (effect of group $F1,15=2.626$, $p=0.0415$ and time $F13,195=2.928$, $p=0.007$) **(Fig. 5a)** in temporal association with reductions in immobility during the exposure to the TST ($p=0.0055$) **(Fig. 5b)**.

**Knockdown of TASK3 in Norepinephrine Neurons Induces Modest Antidepressant-like Effects**

We next examined the effects of Reb-TASK3-siRNA on variables, which are predictive of antidepressant activity. Intranasal Reb-TASK3-siRNA (5.25 nmol/day, 7 days) treatment attenuated the effect of clonidine on decreasing extracellular NE levels in mPFC, suggesting a lower $\alpha_2$-adrenoceptor functional activity **(Fig. 6a)**. Two-way ANOVA showed an effect of group $F_{1,17}=10.12$, $p=0.0055$; time $F_{11,187}=4.466$, $p<0.0001$ and group-by-time interaction $F_{11,187}=2.471$, $p=0.0065$. However, this effect was not translated into a higher baseline NE concentration **(Suppl. Table 2)** nor into an enhanced effect of the NET blocker reboxetine on extracellular NE level in mPFC **(Fig. 6b)**.

Regarding to behavioral paradigms, the selective reduction of TASK3 in NE neurons reduced the immobility time in the TST ($p=0.0032$) **(Fig. 6c)**, but did not affect the performance in the NSFT ($p=0.6319$) **(Fig. 6d)** and MBT ($p=0.9999$) (**Fig. 6e**) as well as in the FST and OF (data not shown).

In addition, Reb-TASK3-siRNA i.n. treatment evoked marginally significant effects on the hippocampal cellular proliferation (Ki-67-positive cells) and BDNF expression in the DG **(Figs. 6f, 6g, 6i)** but, significantly increased BDNF and ARC levels in the CA3 as well as the last in CA1 (**Figs. 6h, 6i**). Two-way ANOVA showed an effect of group $F_{1,28}=15.1$, $p=0.0006$ and $F_{1,28}=27.99$, $p<0.0001$ for BDNF and ARC expression, respectively. Overall, the reduction of TASK3 expression in TH-positive neurons of LC elicited milder antidepressant-like effects than those evoked by TASK3 knockdown in 5-HT neurons.

## Discussion

In this study, we report that siRNA-induced knockdown of TASK3 channel in monoaminergic neurons elicits fast antidepressant-like responses in mice, more marked when TASK3 expression was reduced in 5-HT neurons. The design of Ser- and Reb-conjugated siRNA molecules allowed us to allocate them selectively in 5-HT and NE neurons, respectively, after i.n. administration. Using this strategy, we were able to reduce the expression of TASK3 channels only in these monoaminergic cell groups,

with no signs of neuronal and glial toxicity or compensatory mechanisms involving the expression of other members of $K_{2P}$ channel family as TREK1 and TASK1. These results support TASK3 as a new target for novel antidepressant therapies, which would overcome the limitations of standard antidepressant treatments, including slow clinical action and low efficacy.

Specific accumulation of Ser-conjugated siRNA molecules in 5-HT neurons or of antisense oligonucleotides conjugated with indatraline (triple monoamine reuptake inhibitor) in 5-HT, dopamine (DA) and NE neurons after i.n. administration has been previously reported **[9, 10, 25]**. Here we extend this approach to NE neurons of the LC by covalently binding siRNA molecules to Reb, which allows the selective delivery and internalization of oligonucleotides to NE neurons after systemic (intranasal) administration. Delivery mechanism(s) of the conjugated siRNA molecules to monoamine cell bodies remain still poorly understood. Given the anatomical proximity of DR and LC to the cerebral aqueduct and the fourth ventricle, respectively, conjugated siRNAs may be rapidly transported via CSF by pulsatile flow and then, taken up by the dense network of axons emerging from monoamine cell bodies in both nuclei, which contain the largest densities of SERT and NET in the brain **[33, 34]**. This mechanism is supported by the short time (10-20 min) taken by indatraline-conjugated oligonucleotides to reach the monoaminergic nuclei (DR, *substantia nigra* pars compacta, ventral tegmental area and LC) after i.n. administration, as assessed by microdialysis **[25]**. Moreover, the association of conjugated siRNA molecules with endomembrane Rab family in monoaminergic neurons [**10, 25,** present study] suggests an additional involvement of a complex intracellular trafficking of conjugated oligonucleotides.

Like previous data **[9, 10, 25],** the presence of sertraline or reboxetine in the conjugated siRNA molecules is required for their selective accumulation in 5-HT and NE neurons, respectively, as observed with the double conjugated A488-Ser-NS-siRNA and A488-Reb-NS-siRNA, respectively. However, conjugated NS-siRNA did not modify the TASK3 mRNA expression in DR and LC, respectively, supporting the specificity of the effect.

Ser-TASK3-siRNA administration evoked significant changes in pre- and postsynaptic markers, which are predictive of clinical antidepressant activity. Likewise, the i.n. treatment was effective in the TST, FST and NSFT, used to assess antidepressant-like efficacy in mice, but not in the

MBT, mainly used to assess anxiolytic and anti-obsessive/compulsivity disorder (OCD) behaviors. Interestingly, the sensitivity of NSFT to chronic –but not acute- standard antidepressant administration and to fast-acting treatments, such as ketamine **[30, 35, 36]**, suggests the superior efficacy of short-term Ser-TASK3-siRNA treatments. In addition, since the Ser-NS-siRNA treatment (7 days, i.n.) did not induced any change in TST, NSFT or the hedonic state **[10]**, the antidepressant-like responses found herein should be consequences of downstream changes of reduced TASK3 expression/function in 5-HT (or NE) neurons, but not due to an effect of the minute amounts of sertraline (or reboxetine) contained in the conjugated siRNA. Hence, systemic dose-ranges of 10-20 mg/kg/day (~300-600 µg/day) sertraline or reboxetine are necessary to evoke antidepressant responses in rodents **[37-41]**. However, the dose of sertraline or reboxetine present in the conjugated siRNA was 1.53 µg/day, which represents 200-400 time less than that required to induce antidepressant-like effects.

The antidepressant-like effects of Ser-TASK3-siRNA are likely mediated by an enhancement of forebrain serotonergic neurotransmission associated to a reduced function of 5-$HT_{1A}$-autoreceptors, thus decreasing the efficacy of negative feedback mechanisms operating at somatodendritic level **[42, 43]**. Similarly, the efficacy of the $\alpha_2$-adrenoceptor agonist clonidine to reduce NE release was dampened in Reb-TASK3-siRNA-treated mice, indicating a comparable reduction of $\alpha_2$-adrenoceptor sensitivity. However, unlike fluoxetine in Ser-TASK3-siRNA-treated mice, this was not accompanied by a greater effect of reboxetine in increasing extracellular NE in forebrain, as observed with combinations of NET inhibitors and $\alpha_2$-adrenoceptor antagonists **[44, 45]**. The reduced sensitivity of 5-$HT_{1A}$-autoreceptors and $\alpha_2$-adrenoceptors in mice treated with conjugated-TASK3-siRNA would be causing changes in membrane potential of monoamine neurons after partial TASK3 inactivation. Indeed, a lower number of constitutively active TASK3 channels would increase resting membrane potential **[46]**, thus becoming 5-HT and NE neurons less sensitive to the hyperpolarizing actions of somatodendritic autoreceptors. Alternatively, an intra-membrane interaction between G protein-coupled inwardly rectifying potassium (GIRK) channels associated to monoamine autoreceptors and TASK3 channels could be involved. Supporting this view, previous studies indicated that activating 5-$HT_{1A}$-autoreceptors, which primarily open GIRK channels, hyperpolarizing the cell and reducing firing, also decreases cAMP levels, which may in turn result in a disinhibition of $K_{2P}$ TREK-1

channels –other member of $K_{2P}$ family–, also resulting in hyperpolarization **[47, 48]**. Together, this evidence confirms that $K_{2P}$ channels (TREK-1 and TASK3) play a key role in the regulation of 5-HT and NE neurotransmission.

Moreover, fluoxetine and its metabolite norfluoxetine have been described as potent inhibitors of TREK-1 channel ($IC_{50}$ 19 and 9 µM, respectively) by a mechanism that involved a decreased dissociation of C-terminal domain from the membrane **[49, 50]**. Thereby, SSRIs could potentially inhibit TREK-1 in two ways: directly, and via increasing 5-HT release onto cAMP-inhibiting 5-$HT_{1A}$ receptors. In contrast, fluoxetine has a less potent inhibitory action on TASK1 and TASK3 channels ($IC_{50}$ 100 µM) **[49, 51]**, indicating that the greater effect of fluoxetine on the extracellular 5-HT concentration in TASK3 knowdown mice would be potentially linked to the inhibition of 5-$HT_{1A}$ autoreceptor-dependent negative feedback loop. To our knowledge, no similar data were reported for reboxetine. Further studies are necessary to understand the nature of the interactions between the monoamine transporters (SERT, NET), autoreceptors (5-$HT_{1A}$, $\alpha_2$-adrenoceptor) and TASK3 channel antagonism to evoke more rapid antidepressant response than conventional antidepressant drugs.

As observed after the knockdown of 5-$HT_{1A}$-autoreceptors **[11]**, mice treated with Ser-TASK3-siRNA exhibited a reduced immobility time in the TST, in parallel to an enhanced 5-HT release. The similarity of both effects suggests a common underlying mechanism: both treatments would reduce self-inhibitory inputs onto 5-HT neurons during stressful conditions, thus enhancing serotonergic activity in forebrain and increasing resilience to stress. Conversely, an increased expression/function of 5-$HT_{1A}$-autoreceptors is associated with poor antidepressant efficacy and increased suicidal behavior **[52-55]**, and mice with a high expression of 5-$HT_{1A}$-autoreceptor show a depressive-like phenotype **[56]**. Overall, these observations support a direct relationship between reduction of self-inhibitory mechanisms in 5-HT neurons and antidepressant activity, to which a reduced TASK3 expression/function can contribute in a significant manner, similarly to what was reported with the 5-$HT_{1A}$-autoreceptor knockdown **[9, 11, 56]**.

Along with previous presynaptic changes, Ser-TASK3-siRNA and Reb-TASK3-siRNA increased hippocampal neurogenesis (Ki-67- and DCX-positive newborn cells) and the expression of plasticity genes (BDNF, ARC, VEGF). These effects, produced in 3-4 weeks with standard

antidepressant treatments **[11, 57-59]**, required only a 7-day treatment with Ser-TASK3-siRNA. TASK3 knockdown in 5-HT and NE neurons might act more quickly to alleviate depression, particularly because it does not a require desensitization of presynaptic autoreceptors **[43]** or an increase in neurogenesis **[60]**, two of the leading hypotheses to explain the delayed onset of action of SSRIs. Moreover, this faster action was also observed with unmodified and conjugated SERT-siRNA **[10, 12]** and likely reflects the greater effectiveness of RNAi strategies to modulate neuronal function, compared to standard pharmacological treatments.

A key observation of the present study is that behavioral and neurochemical effects evoked by Reb-TASK3-siRNA were less marked than those evoked by Ser-TASK3-siRNA, and in some instances, did not reach statistical significance. This difference may be attributable to different factors. On the one hand, a lesser ability of unmodified and conjugated TASK3-siRNA to reduce TASK3 expression in NE neurons of LC (84 and 76%, respectively) compared to DR 5-HT neurons (69 and 60%, respectively). In the case of the conjugated siRNA, this difference might also be associated with the higher affinity of sertraline for SERT than reboxetine for NET [61, 62]. On the other hand, 5-HT and NE likely play differential roles in the treatment of MDD symptoms, being the 5-HT system more deeply involved in resilience to stress, a key factor in the performance of the behavioral tests used (TST and NSFT).

In summary, the present study shows that the selective reduction of TASK3 expression in monoamine neurons evokes antidepressant-like effects, being more significant when it targets 5-HT neurons than NE neurons. One-week treatments with Ser-TASK3-siRNA evoked behavioral and neurobiological changes comparable to those produced by prolonged SSRI treatments (e.g. one-month). These effects may be driven by a reduced sensitivity of monoamine neurons to self-inhibitory inputs after TASK3 knockdown, thus enhancing monoamine neurotransmission. Further, the extension of the conjugated-siRNA strategy from 5-HT to NE neurons supports the validity of the present approach as a new therapeutic strategy for MDD treatment.

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## Conflict of Interest

F.A. has received consulting honoraria on antidepressant drugs from Lundbeck and he has been PI of grants from Lundbeck. A.B. has been PI of grants from Nlife Therapeutics S.L. F.A. and A.B. are co-authors of the patent WO/2011/131693 for the siRNA and ASO (antisense oligonucleotides) molecules and the targeting approach related to this work. The rest of authors declare no competing financial interest.

## Additional files

Supplementary tables: 2
Supplementary figures: 6

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## Figures Legends

**Figure 1.** Selective accumulation of sertraline- or reboxetine-conjugated nonsense siRNA (Ser-NS-siRNA or Reb-NS-siRNA) in serotonin (5-HT) and norepinephrine (NE) neurons after intranasal administration. Mice were intranasally administered with: 1) alexa488-PBS (A488-PBS), 2) alexa488-labeled nonsense siRNA (A488-NS-siRNA) or 3) alexa488-labeled Ser- or Reb-NS-siRNA (A488-Ser-NS-siRNA or A488-Reb-NS-siRNA) at 2.1 nmol/day during 4 days, and were sacrificed 6 h post-administration (n = 2 mice/group). **a, b** Laser confocal images showing the co-localization (yellow) of A488-Ser-NS-siRNA or A488-Reb-NS-siRNA in 5-HT neurons in the dorsal raphe nucleus (DR) identified with a $TPH_2$ marker antibody (red) **(a)** or in NE neurons of the *locus coeruleus* (LC) identified with TH marker antibody (red) **(b)**. White arrowheads show the siRNA molecules co-localized with $TPH_2$- and TH-positive cells. However, neither A488-Ser-NS-siRNA was detected in TH-positive cells in LC **(a)** nor A488-Reb-NS-siRNA in $TPH_2$-positive cells in DR **(b)** indicating the selective incorporation of the oligonucleotides in each monoamine neuronal group expressing the corresponding transporters. Cell nuclei were stained with Dapi (blue). Scale bars: 10 μm. **c** Confocal images showing the co-localization (yellow) of A488-Ser-NS-siRNA or A488-Reb-NS-siRNA (green) with Rab7 (red) in DR or LC neurons. Vesicles are marked with white arrowheads. The boxes include areas of DR or LC shown at higher magnification Scale bars: low = 10 μm and high = 5 μm

**Figure 2.** Intranasal sertraline-conjugated TASK3-siRNA (Ser-TASK3-siRNA) treatment down-regulates TASK3 expression in mouse serotonin (5-HT) neurons. Mice received: 1) PBS or 2) Ser-TASK3-siRNA at 5.3 nmol/day for 7 days, and sacrificed 3-4 days after last administration. **a** Photomicrographs showing $TPH_2$-positive neurons expressing TASK3 mRNA ($^{33}$P-oligonucleotide silver grains) in the dorsal raphe nucleus (DR) at two anteroposterior (AP) coordinates from bregma (AP1: -4.24 to -4.48 and AP2: -4.48 to -4.72 in mm) of PBS- and Ser-TASK3-siRNA-treated mice. Scale bar: 10 μm. **b, c** Autoradiographic analysis showed that Ser-TASK3-siRNA reduces TASK3 mRNA levels in the DR **(b)**, but not in forebrain areas such as CA1, dentate gyrus (DG) and medial

prefrontal cortex (mPFC) **(c)** (n = 4-6 mice/group). **d** Intranasal Ser-TASK3-siRNA treatment reduced the percentage of $TPH_2$-positive neurons expressing TASK3 mRNA in the DR of mice (n = 4-6 mice/group). However, Ser-TASK3-siRNA did not modify the number of DR $TPH_2$-positive neurons of the same mice (showed in **Suppl. Fig. 5a**). **e** Dipping analysis also revealed a reduction of intracellular TASK3 mRNA density in $TPH_2$-positive neurons of DR (n = 4-6 mice/group). **f** Western blotting of mPFC, hippocampus (HPC) and DR showing TASK3 and actin protein levels. Actin was used as loading control. Note the decreased TASK3 protein density in the DR of Ser-TASK3-siRNA-treated mice. **g** Relative quantification of TASK3 protein level obtained by normalizing TASK3 by actin protein amount (n = 5-7 mice/group). Data are presented as the mean ± SEM. *p<0.05, **p<0.01 compared to control group treated with PBS.

**Figure 3.** Intranasal reboxetine-conjugated TASK3-siRNA (Reb-TASK3-siRNA) treatment down-regulates TASK3 expression in mouse norepinephrine (NE) neurons. Mice were treated with: 1) PBS or 2) Reb-TASK3-siRNA at 5.3 nmol/day for 7 days, and sacrificed 3-4 days after last administration. **a** Photomicrographs showing TH-positive neurons expressing TASK3 mRNA ($^{33}$P-oligonucleotide silver grains) in the *locus coeruleus* (LC) at two anteroposterior (AP) coordinates from bregma (AP1: -5.52 to -5.68 and AP2: -5.68 to -5.80 in mm) of PBS- and Reb-TASK3-siRNA-treated mice. Scale bar: 10 μm. **b, c** Autoradiographic analysis showed that Reb-TASK3-siRNA reduces TASK3 mRNA levels in the LC (B), but not in forebrain areas such as CA1, dentate gyrus (DG) and, medial prefrontal cortex (mPFC) (C) (n = 8 mice/group). **d** Intranasal Reb-TASK3-siRNA treatment reduced the percentage of TH-positive neurons expressing TASK3 mRNA in LC of mice (n = 4-6 mice/group). However, Reb-TASK3-siRNA did not modify the number of LC TH-positive neurons of the same mice (showed in **Suppl. Fig. 5a**). **e** Dipping analysis also revealed a reduction of intracellular TASK3 mRNA density in TH-positive neurons of LC (n = 4-6 mice/group). **f** Western blotting of mPFC, hippocampus (HPC) and LC showing TASK3 and actin protein levels. Actin was used as loading control. Note the decreased TASK3 protein density in the LC of Reb-TASK3-siRNA-treated mice. **g** Relative quantification of TASK3 protein level obtained by normalizing TASK3 by actin protein

amount (n = 6 mice/group). Data are presented as the mean ± SEM. ^p< 0.05, ^^p<0.01, ^^^p<0.001 compared to control group treated with PBS.

**Figure 4.** Sertraline-conjugated TASK3-siRNA (Ser-TASK3-siRNA) evokes neurochemical, behavioral and cellular responses predictive of clinical antidepressant activity. Mice received intranasally 1) PBS or 2) Ser-TASK3-siRNA at 5.3 nmol/day for 7 days and were sacrificed 3-4 days after last administration. **a** Ser-TASK3-siRNA treatment reduced the effect of serotonin-1A ($5\text{-}HT_{1A}$) receptor 8-OH-DPAT agonist (1 mg/kg, i.p.) on serotonin (5-HT) release in medial prefrontal cortex (mPFC) (n = 9-10 mice/group). **b** Fluoxetine (20 mg/kg, i.p.) increased the extracellular 5-HT concentration significantly more in Ser-TASK3-siRNA than in PBS-treated mice (n = 6 mice/group). **c, d** Ser-TASK3-siRNA treated mice displayed reduced immobility in the tail suspension test (TST, n = 11 mice/group) and forced swim test (FST, n = 7-10 mice/group). **e** Effect on novelty suppressed feeding test (NSFT) and survival analysis. In two cohorts, DR TASK3 knockdown mice showed a better performance in the NSFT compared to control groups (n = 9-12 mice/group/cohort). **f** PBS and Ser-TASK3-siRNA-treated mice behaved similarly in the marble burying test (MBT, n = 13-14 mice/group). **g** Representative images showing Ki-67- or DCX-positive cells in the dentate gyrus (DG) of Ser-TASK3-siRNA- or PBS-treated mice. Scale bar: 100 μm. **h** Ser-TASK3-siRNA significantly increased the number of Ki-67- and DCX-positive cells compared to the PBS group (n = 5-7 mice/group). **i** Representative autoradiograms showing BDNF, ARC and VEGF mRNA expression in the hippocampus of control and TASK3 knockdown mice. Scale bar: 100 μm. **j** Densitometric analyses of BDNF, ARC and VEGF mRNA level were performed in different hippocampal regions: CA1, CA2, CA3 and dentate gyrus (DG) shown in the cresyl violet-stained section (left bottom) (n = 5-6 mice/group). Data are presented as the mean ± SEM. *p<0.05, **p<0.01 and ****p<0.0001 versus PBS-treated mice.

**Figure 5.** Sertraline-conjugated TASK3-siRNA (Ser-TASK3-siRNA) treatment enhanced serotonin (5-HT) release in the medial prefrontal cortex (mPFC) of mice under short-term inescapable stress paradigm. Mice received intranasally 1) PBS or 2) Ser-TASK3-siRNA at 5.3 nmol/day for 7 days and were evaluated 2-3 days after last administration. **a** During a stressful situation induced by the tail suspension test (TST), TASK3 knockdown mice showed a larger increase of extracellular 5-HT in mPFC than PBS-treated mice (n = 8-9 mice/group). **b** Simultaneously, Ser-TASK3-siRNA-treated mice displayed a reduced immobility in the TST (*n* = 8-9 mice/group). Data are presented as the mean ± SEM. *p<0.05 and **p<0.01 versus PBS-treated mice.

**Figure 6.** Mild antidepressant-like effects produced by reboxetine-conjugated TASK3-siRNA (Reb-TASK3-siRNA). Mice received intranasally 1) PBS or 2) Reb-TASK3-siRNA at 5.3 nmol/day for 7 days and were sacrificed 3-4 days after last administration. **a** Reb-TASK3-siRNA treatment reduced the effect of $\alpha_2$-adrenoreceptor clonidine agonist (0.3 mg/kg, i.p.) on extracellular norepinephrine (NE) levels in medial prefrontal cortex (mPFC) (n = 9-10 mice/group). **b** Reboxetine (20 mg/kg, i.p.) increased the extracellular NE levels similarly in both experimental groups (PBS and Reb-TASK3-siRNA) (n = 8 mice/group). **c** Reb-TASK3-siRNA treated mice displayed a reduced immobility in the tail suspension test (TST) versus PBS-treated mice (n = 9-11 mice/group). **d** In two cohorts, both PBS and Reb-TASK3-siRNA mice behaved similarly in the novelty suppressed feeding test (NSFT) (n = 9 mice/group/cohort). **e** No anxiety-related effects were observed on the marble burying test (MBT) (n = 12 mice/group). **f** Representative images showing Ki-67- or DCX-positive cells in the dentate gyrus (DG) of Reb-TASK3-siRNA or PBS-treated mice. Scale bar: 100µm. **g** Reb-TASK3-siRNA did not induce any increase in proliferation (Ki-67-positive cells) or neurogenesis (DCX-positive cells) in DG compared to the PBS group (n = 6 mice/group). **h** Representative autoradiograms showing BDNF, ARC and VEGF mRNA expression in the hippocampus of control and TASK3 knockdown mice. Scale bar: 100µm. **i** Densitometric analyses of BDNF, ARC and VEGF mRNA density were performed in different hippocampal regions: CA1, CA2, CA3 and dentate gyrus (DG) shown in the

cresyl violet-stained section (left bottom) (n = 4-5 mice/group). Data are presented as the mean ± SEM. ^p<0.05 and ^^p<0.01 versus PBS-treated mice.

Figure 1

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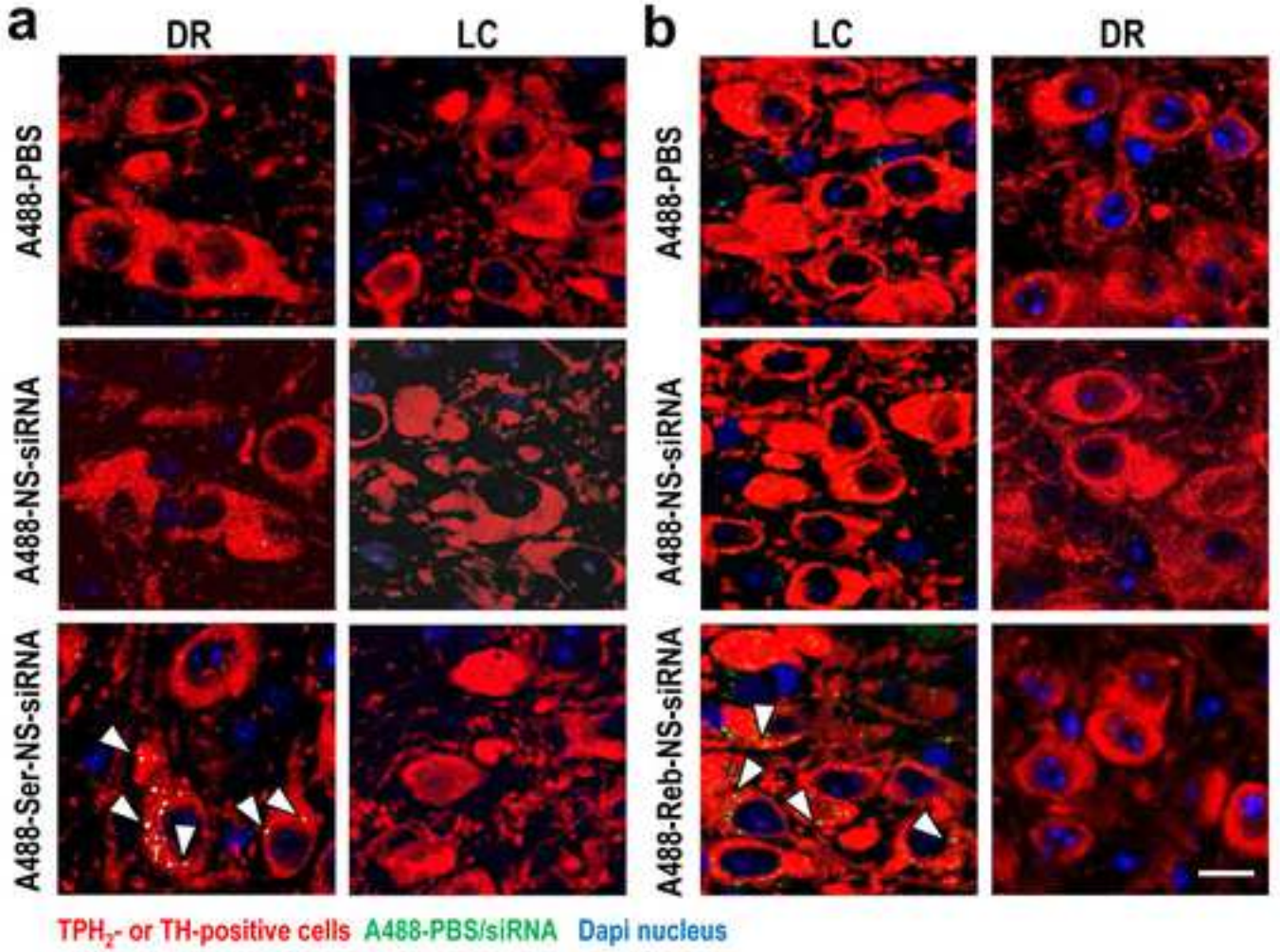


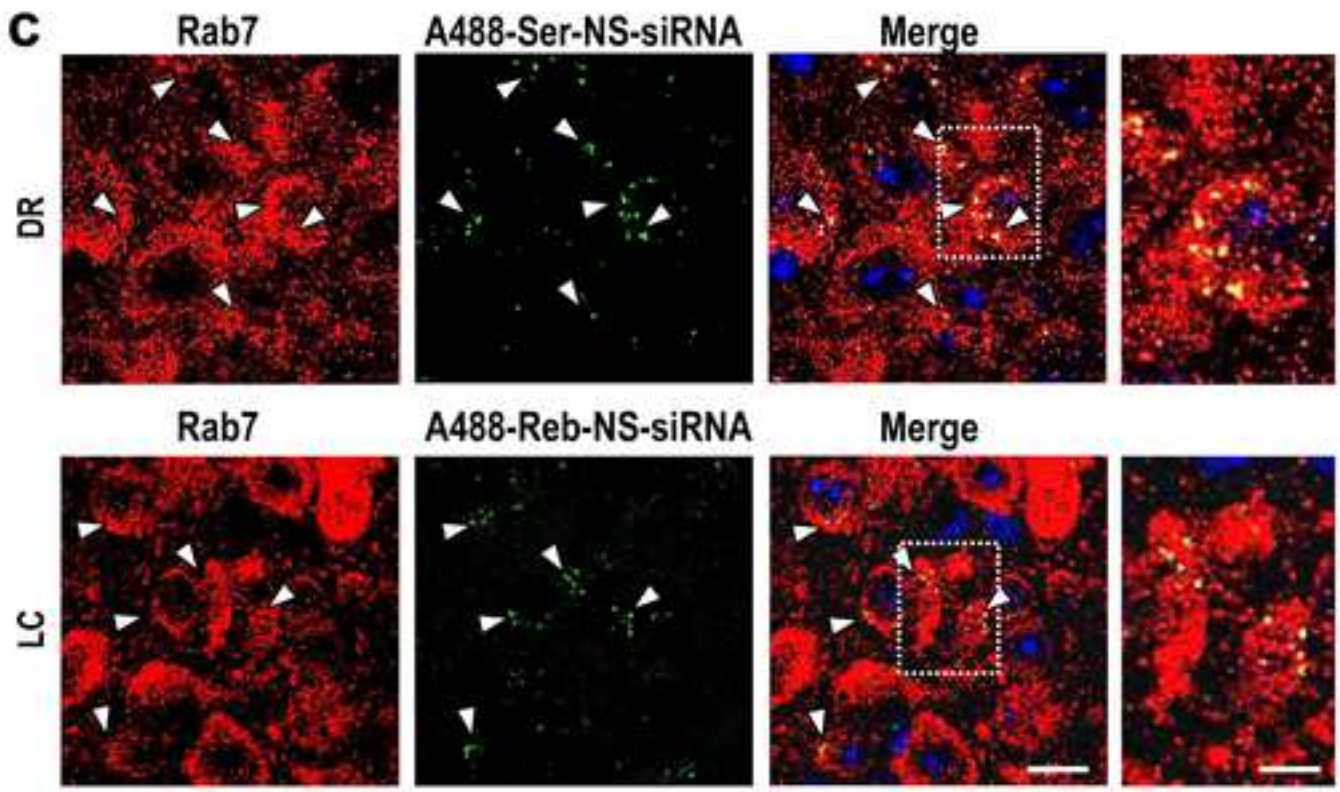

Figure 2

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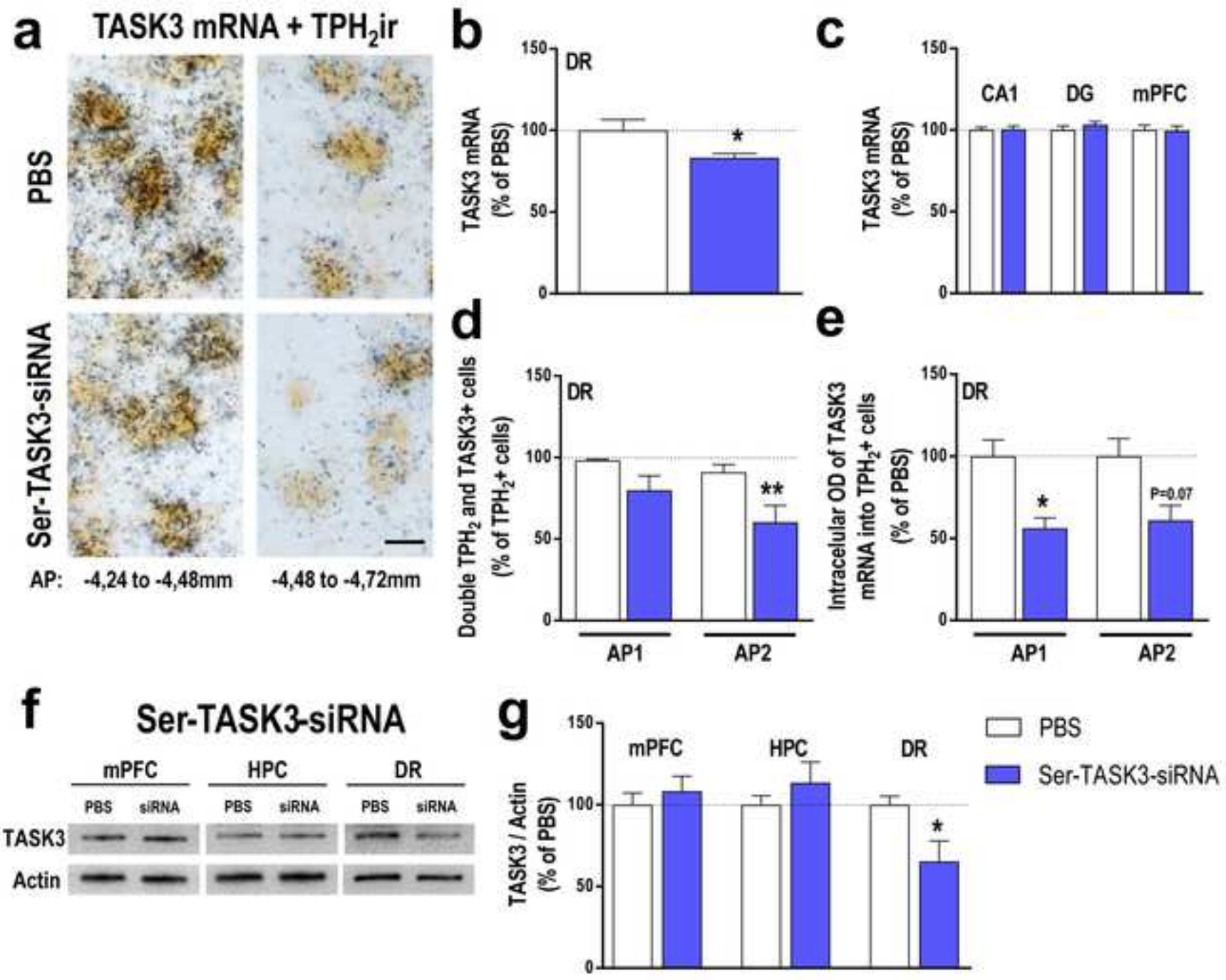

Figure 3

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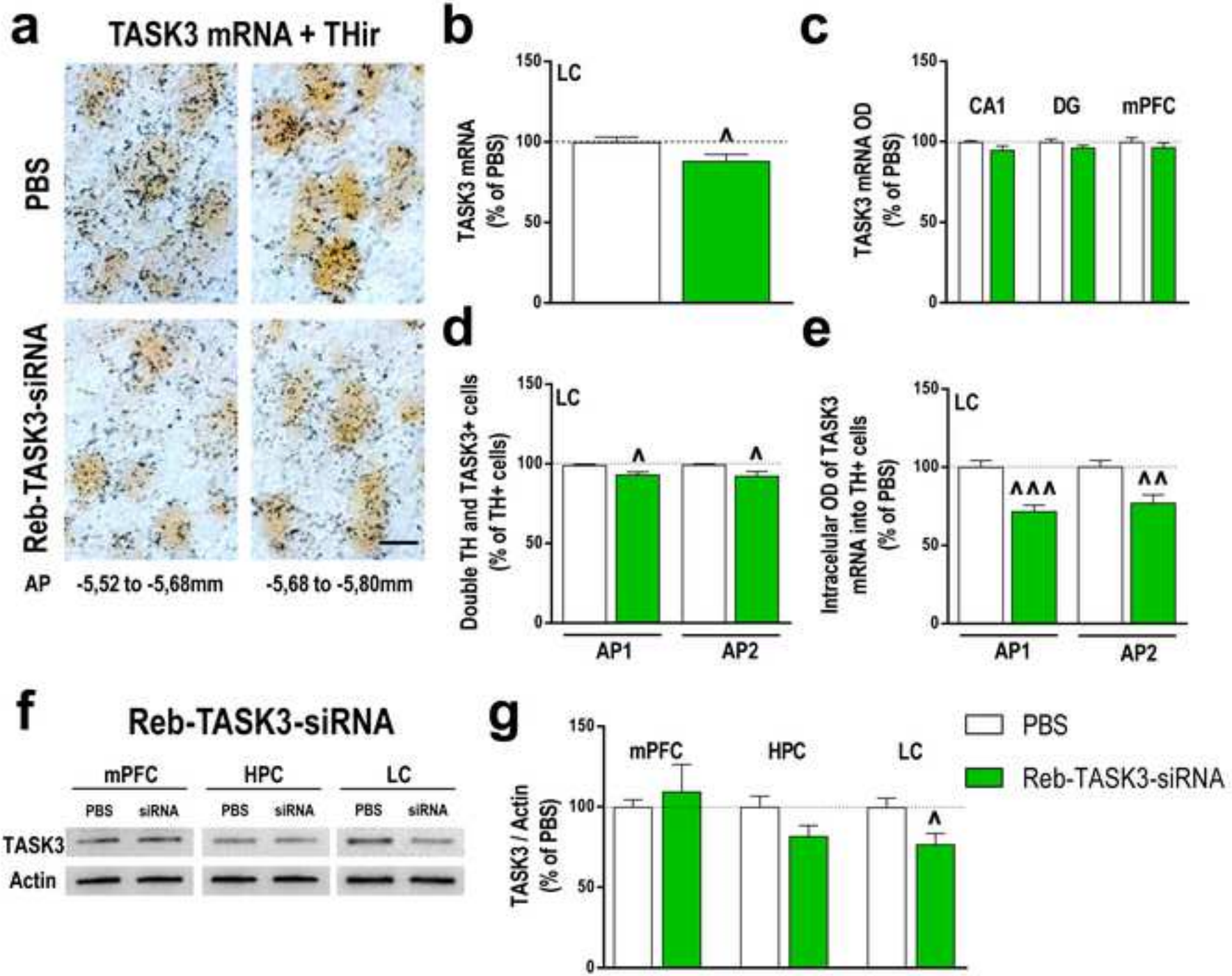

Figure 4

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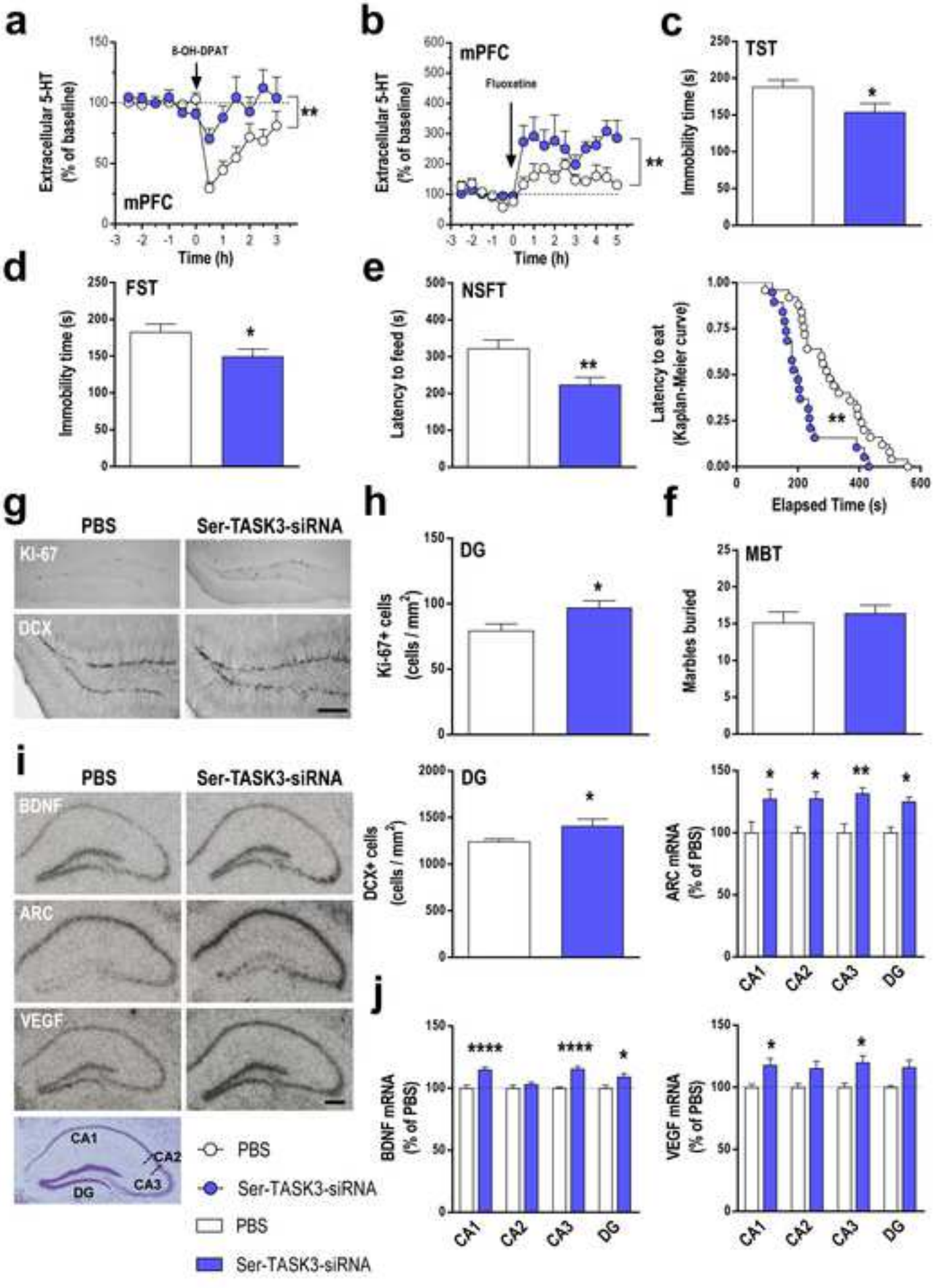

Figure 5

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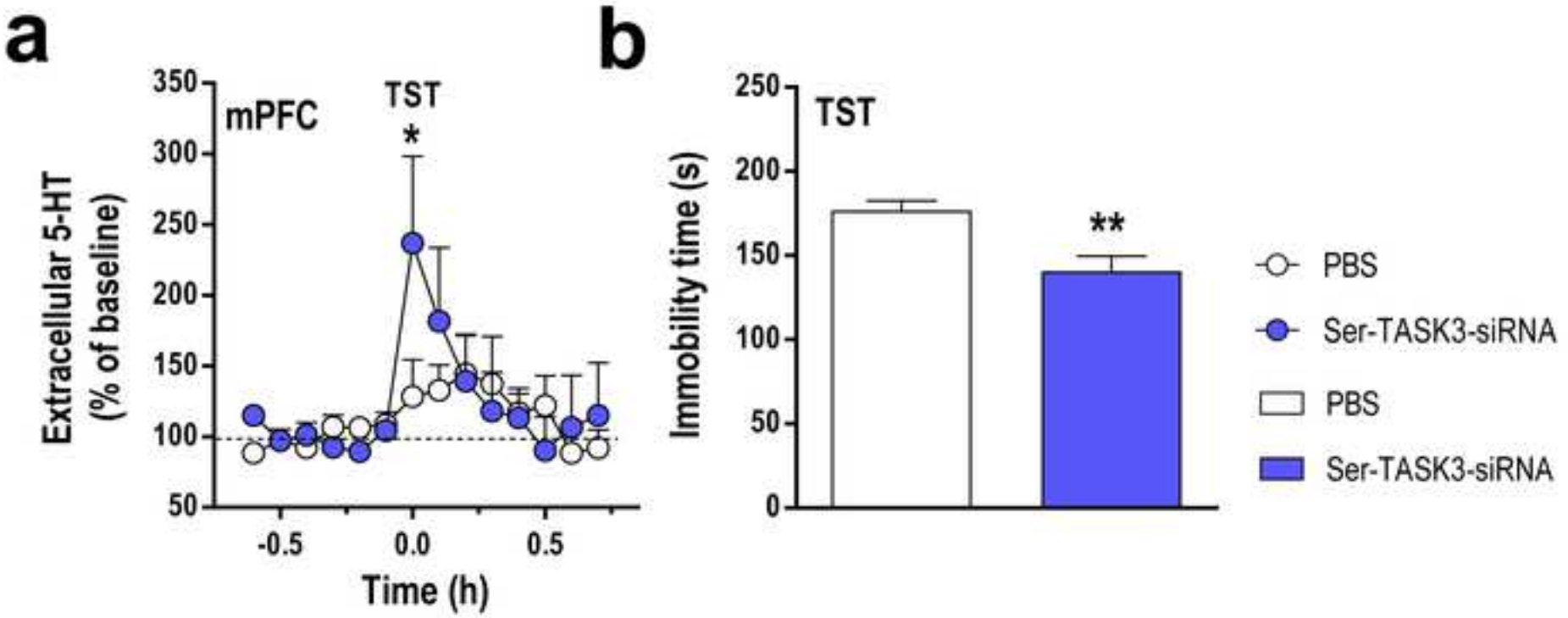

Figure 6

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