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Abstract: We have investigated the relevance of D-Aspartate Oxidase (DDO), the only enzyme known to selectively degrade D-Asp, in modulating glutamatergic system homeostasis. Interestingly, the lack of the Ddo gene, by raising D-Asp content, induces a substantial increase in extracellular glutamate (Glu) levels in Ddo mutant brains. Consistent with an exaggerated and persistent NMDA receptor (NMDAR) stimulation, we documented in Ddo knockouts severe age-dependent structural and functional alterations mirrored by expression of active caspase 3 and 7 along with appearance of dystrophic microglia. In addition, elevated D-Asp content triggered in mutants alterations of NMDAR-dependent synaptic plasticity associated to reduction of hippocampal GluN1 and GluN2B subunits selectively located at synaptic sites. These effects, all of which converged on a progressive hypo-responsiveness at NMDAR sites, functionally resulted in a greater vulnerability to phencyclidine-induced prepulse inhibition deficits in mutants. In conclusion, our results indicate that DDO, by strictly regulating D-Asp levels, impacts on the homeostasis of glutamatergic system thus preventing accelerated neurodegenerative processes.

September 22nd, 2014

Dear Editor,

Please find enclosed a manuscript entitled "D-aspartate-oxidase influences glutamatergic system homeostasis in an age-dependent fashion in mammalian brain" by Cristino *et al.*, which my co-authors and I would like to be considered for publication in *Neurobiology of Aging* as an original contribution, that has not been previously published and submitted for publication elsewhere while under consideration.

Free D-aspartate (D-Asp) is present in markedly high amounts in the embryonic brain of mammals. After birth, the endogenous levels of this atypical amino acid rapidly decrease due to the onset of its catabolic enzyme, D-Aspartate Oxidase (DDO). The work here presented follows two articles that we published in *Neurobiology of Aging* showing that D-Asp, by activating NMDARs through the binding to the glutamate site of GluN2 subunits (Errico F, et al (2011) Neurobiol Aging 32:2229–2243), modulates a number of NMDAR-mediated processes including synaptic plasticity and memory (**Errico F, et al (2011) Neurobiol Aging 32:2061–2074).** Moreover, our group has recently shown that deregulated higher D-Asp brain content in mice is associated with augmented spine density and dendritic length in the hippocampus and prefrontal cortex (PFC) (Errico F, et al (2014) Transl Psychiatry 29:4:e417). In keeping with animal data, a single nucleotide polymorphism (SNP) within the human *DDO* gene predicts reduced expression of *DDO* in the *post-mortem* PFC, and is associated with greater prefrontal grey matter and activity during working memory in healthy subjects (Errico F, et al. Transl Psychiatry 29:4:e417). Collectively, the close control exerted by D-Asp on NMDAR function and its neurodevelopmental occurrence allow the hypothesis that this amino acid is a potential pathophysiological mediator of some of the NMDAR-related alterations observed in SCZ. Consistently, we recently described a substantial reduction of D-Asp and of its N-methyl derivative, NMDA, in *post-mortem* PFC of schizophrenic patients, compared to healthy controls (Errico F, et al. J Psychiatr Res. 2013 Oct; 47:1432-7).

On the other hand, it is still unknown the influence of DDO in controlling glutamatergic system homeostasis. Here we use a multidisciplinary approach to provide new

mechanistic insights reporting that lack of the *Ddo* gene, by raising D-Asp content, induces a substantial increase in extracellular glutamate (Glu) levels in mutant brain. Moreover, we demonstrate that the application of D-Asp on synaptosomes stimulates Glu release, and that acute injection of this D-amino acid increases Glu levels in the prefrontal cortex of freely moving mice. Interestingly, high and persistent D-Asp and Glu content in 6-month-old *Ddo* mutant brains produced progressive alterations of the excitatory glutamatergic inputs in the hippocampus and cortex, which was paralleled by neuroinflammatory processes as indicated by the appearance of dystrophic microglia. Furthermore, non-physiological exposure to elevated D-Asp and Glu levels in *Ddo* mutant brain triggered a profound reduction in hippocampal long-term potentiation and in the GluN1 and GluN2B subunits of NMDAR located at synaptic sites. These effects, all of which converged on hypo-glutamatergic responsiveness at NMDAR sites in 6-month-old *Ddo* mutants, functionally resulted in a greater sensitivity to phencyclidine-induced prepulse inhibition deficits. Taken together, our results indicate that *Ddo* gene profoundly impacts on the homeostasis of the glutamatergic system and its dysregulation might accelerate age-dependent NMDA-related processes in the mammalian brain.
Based on the above observations, we hope you will find this manuscript of interest for the readers of *Neurobiology of Aging*.

I declare that all authors have no competing financial interests in relation to the work described.

Best regards,
Alessandro Usiello, PhD

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Dear Dr. Rapp,

Thank you for your e-mail message of November 14 with the comments of the referees, which were very helpful. In summary, Reviewer 1 agreed that the manuscript offers novel data that are of considerable interest, but enumerated some points that have been all addressed. Concerns of the Reviewer 2 were mainly based on the interpretation of the results and few methodological aspects. We have now revised the manuscript according to all the comments raised in the two critiques (cf. below) and also added three new figures. In particular we show the presence of reactive astrocytes (new Fig. 4) along with increased caspase 3 and 7 expression (new Fig. 5) in the hippocampus and PFC of *Ddo*-KO mice at six-month of age. In addition, we carried out an extensive electrophysiological analysis showing age-related changes in the AMPAR/NMDAR ratio in *Ddo* mutant brains (new Fig. 7). These new pieces of evidence further strengthen the role of *Ddo* gene in regulating glutamatergic system homeostasis. We hope that with these changes the manuscript is now acceptable for publication in *Neurobiology of Aging*.

Sincerely,

Alessandro Usiello

Reviewer 1

In the present elegant study, data are presented using well-controlled and multiple complementary technical approaches showing that long lasting increase in glutamate availability induced by DDO deletion produces age-dependent alterations in morphology (density of excitatory synapses, expression levels of synaptic NMDA subtype of receptor) and functionality (magnitude of synaptic plasticity) within cortical and hippocampal glutamatergic systems that affect the response to prepulse inhibition, a measure of sensorimotor gating closely related on NMDAR activation. The results presented here strengthen the idea of a neuroprotective role of DDO on NMDAR-related brain functions through the selective control of D-aspartate concentrations.

Considering the relevance of this result and because the methodology appears to be well thought out and sound and the data clearly presented in the manuscript, this study certainly deserves to be published although some points need to be addressed and /or clarified.

In this study, we don't know the time course and magnitude of the glutamate increase in KO animals since measures presented in fig 1 were obtained only from 6 months-old mice. But if we consider that glutamate levels are also enhanced at 3 months (because the authors previously published that D-aspartate concentrations were 15-20 fold higher than WT at this time point), how can it be explained that non-NMDA receptor-mediated synaptic activities remain unaffected (see Errico et al, 2011).

R. We thank the Referee for this very pertinent observation. However, in the previous work (Errico et al, Neurobiology of Aging 32 (2011) 2061–2074) we report a significant reduction in the input-output curves of *Ddo*-KO vs. *Ddo*-WT mice. This type of experiment measures basal synaptic transmission that is mainly mediated by AMPA receptors, thus suggesting that also non-NMDA receptors are affected and might contribute to precocious brain aging processes seen in mutants.

Also, it is shown here that D-aspartate increases glutamate levels by acting on presynaptic receptors. It is therefore surprising that the authors did not previously find changes in paired-pulse facilitation (PPF) (Errico et al, 2008) knowing that PPF is generally used to consider presynaptic effects of a drug.

R. We agree with the referee's observation. In fact, previous experiments carried out in *Ddo*-KO mice never showed any PPF alteration at all age-stages tested (Errico et al, Neurobiology of Aging 32 (2011) 2061–2074). However, different methodological approaches might explain such discrepancy. For example, the two approaches might diverge in the different presynaptic release-protocols adopted that might possibly reflect different presynaptic mechanisms involved. In fact, on the one hand in the electrophysiological experiments we evoke a fEPSP by local stimulation of the Schaffer collaterals through a stimulating electrode, on the other in the synaptosome preparation we apply a 15 mM K^+-stimulation (90 sec pulse) to evoke neurotransmitter outflow. Next, the unaltered PPF observed in *Ddo*-KO mice might also reflect adaptive compensatory changes due to the gene-targeting approach, compared to the acute effect mediated by direct D-Asp application on the synaptosome preparation.

Considering that excitatory synapse density (figure 2) and expression of NMDAR (figure 4) are significantly reduced in 3 months-old KOs, how the increase in LTP expression can be interpreted?

R. We are grateful to the referee for pointing out this important issue. Indeed we argue that the increased NMDAR-dependent LTP found in 3 month-old *Ddo*-KO mice along with the significant reduction in synaptic NMDAR subunit expression, represents the direct consequence of enhanced glutamatergic release in the hippocampus of mutants. Moreover, in line with this hypothesis, the AMPAR/NMDAR ratio is significantly reduced in 3 month-old *Ddo*-KO vs. control mice (see new Fig. 7B).

In addition, figure 4B shows that levels of GluN1 and GluN2 subunits that mainly governs LTP expression, tends to increase between 3 and 6 months of age whereas LTP magnitude decreases at the same period indicating that a correlation between changes in density of NMDAR subunits and those of LTP expression is not very clear.

R. In our manuscript, differently to what erroneously mentioned by the referee "…*that levels of GluN1 and GluN2 subunits that mainly governs LTP expression, tends to increase between 3 and 6 months of age…"* we show a significant progressive decrease of the synaptic NMDARs (see new Fig. 6B), mainly composed by GluN1/GluN2B subunits between 3 and 6-months of age, and more robustly at 12 months in *Ddo*-KO compared to age-matched controls. NMDAR-dependent LTP was reduced from 6 months of age onwards in line with the reduced levels of GluN1 and GluN2 subunits and with the increased AMPAR/NMDAR ratio observed in mutants (see new Fig. 7D).

As a general observation, the authors frequently discussed about alterations of NMDAR activation in their experiments just by considering results from LTP experiments but pure NMDAR synaptic activities (synaptic currents or potentials) have never been isolated and compared at different time after DDO deletion that would certainly strengthen their results. In such a context, it should be important to show that age-related changes in the sensitivity to phencyclidine-induced PPI deficits in KO mice really correlate to alterations of isolated NMDA activities.

R. We thank the referee for his/her helpful comment. Indeed, in the revised version of this manuscript we recorded both AMPAR and NMDAR-isolated activities. In the new Fig. 7 we carried out an extensive electrophysiological analysis using whole-cell voltage-clamp recordings from CA1 pyramidal neurons. No differences were evident in current–voltage (I-V) relationship neither in the excitatory postsynaptic currents mediated by AMPAR nor by NMDAR at 0.5-, 3- and 6-month-old *Ddo*-KO compared to age-matched controls. On the other hand, and in line with biochemical biochemical experiments, an age-related analysis in *Ddo*-KO mice showed that the

AMPAR/NMDAR ratio was progressively increased between 0.5 and 6 months of age. Altogether, these electrophysiological data indicate, as correctly predicted by the referee, an age-dependent dysregulation of AMPAR or NMDAR-mediated expression following persistent elevated D-Asp levels.

3) The section regarding the role of microglia is interesting but rather speculative at this point. Treatment of 6 months-old KOs to prevent the dystrophic phenotype that will rescue glutamate-related plasticity will perfectly validate the author' speculation. Furthermore, since changes in LTP expression, are much more salient at 12 months-old KO mice (see figure 4), did the authors look at microglia phenotype at this age? On the other hand, it is not clear why the astrocyte phenotype has not been considered in this model regarding 1) the pivotal role of these glial cells in glutamate homeostasis (re-uptake by specific transporters but also release of the amino acid), 2) the contribution of these cells to functional plasticity and 3) their role in pathologies related to glutamate deregulation.

R. We agree with the referee about the importance of showing also the astrocyte phenotype. In fact in new Fig. 4 we report the occurrence of significant alteration in astrocytes morphology and distribution in the *Ddo*-KO brains, at the age of 6 months. On the other hand, pharmacological treatment of 6-month-old *Ddo*-KOs with antioxidant compounds (i.e. reducing agents such as N-acetyl-L-cystein) to prevent inflammation and the occurrence of dystrophic phenotype, as well as analysis of microglia phenotype at 12 months old *Ddo*-KO mice require future experiments and are beyond the scope of the present work. However, to further support a neuroprotective role of DDO enzyme, we also present new data showing the appearance of active caspase 3 and 7 expression in the hippocampus and PFC of 6-month-old *Ddo*-KO mice, that unequivocally support the presence of inflammatory events caused by deregulation of D-Asp levels.

Minor points

"Have the authors any idea to explain why the density of asymmetric synapses in 0.5 month-old KOs is so elevated?"

R. This phenomenon could be interpreted as a selective effect of *Ddo* deletion during the early post-natal brain development, when starts its gene expression. According to this hypothesis, we found that the density of both excitatory and inhibitory synapses in CA1 or IL/PL cortex does not change in *Ddo*-WT matched controls (see Fig. 2Q). Furthermore, the presence of higher glutamate levels in *Ddo*-KO could underlie the onset of supernumerary excitatory synapses, in comparison to age-matched WT mice.

** It is shown that presynaptic effects of D-aspartate and NMDA were both antagonized by MK 801. But are the effects of these amino acids also mutually occlusive?*

R. We thank the Reviewer for this valuable input. Unfortunately, we cannot predict whether a combination of D-aspartate and NMDA results in occlusive or additive effects. Indeed, NMDA is a heteromeric receptor, whose GluN2 subunit composition determines its pharmacological properties. MK-801 binds with equal affinity to both GluN2A and GluN2B subunits receptor, so it cannot differentiate between NMDA receptor subpopulations. NMDA binds with equal affinity to different NMDA receptor populations (Laurie and Seeburg, Eur J Pharmacol, 1994) but we do not know whether D-aspartate behaves in the same way, since we did not use NMDA subtype specific antagonists. Therefore, we cannot predict whether the combination of NMDA with D-Aspartate will result in occlusive or additive effects.

Reviewer #2: This manuscript describes research on the effect of knock-down of the D-aspartate oxidase enzyme (Ddo-KO) on characteristics of the N-methyl-D-aspartic acid receptor (NMDAR). The authors have a considerable tract record in this area through their publications.
In this study the authors investigated the effect of Ddo-KO on glutamate release, synapse morphology, LTP, NMDAR subunit expression and prepulse inhibition.

Lack of the Ddo gene, by raising D-Asp content, induces a substantial increase in extracellular glutamate (Glu) levels in Ddo mutant brains.
It is not clear why different units are used in Figs 1A and 1C
R. We thank the Reviewer for these valuable suggestions. We have uniformed the measure units on the Y-axes of the different microdialysis figures.

It cannot be concluded from these experiments that "administration of D-Asp modulates Glu neurotransmission in PFC" without taking into account effects on excitatory amino acid transporters.
R. We thank the Reviewer for this very pertinent question. Glu transporters play indeed a great role in determining extracellular brain levels of D-Asp. It is true that measuring D-asp content may have provided more complete information on the overall D-Asp pool (intracellular + extracellular). Future studies are mandatory to decipher this issue. On the other hand, experiments in cortical synaptosomes were always performed in the presence of the transporter inhibitor DL-TBOA thus excluding any reversal effect of Glu transporter in the action triggered by D-Asp on Glu release.

Age-dependent reduction of excitatory synaptic input to the pyramidal neurons of the hippocampal CA1 field and of the IL/PL cortex in Ddo-KO brain.
In this section, the authors show a robust decrease in asymmetric synapses CA1 of hippocampus in Ddo-KO at 6 months compared 0.5 months. No similar decrease was found in WT-animals. There appear to be inconsistencies in the description in the final part of this section that need clarification.
R. About this point the authors refer now to new set of data concerning the strong expression of active-caspase-3 and caspase-7 immunoreactivities, which most likely underlies the occurrence of remodeling synaptic processes and apoptotic events in numerous pyramidal neurons of 6-month-old *Ddo*-KO compared to age-matched controls and younger *Ddo*-KO mice (see new Fig. 5).

Age-dependent changes in CA1-LTP and synaptic NMDAR subunit composition in the hippocampus of Ddo-KO mice
Differences in LTP between wild-type and Ddo-KO mice were only seen at 3 months and 12 months and not at 0.5 and 6 months which led the authors to analyse levels of expression of the NMDAR subunits. More information is needed about the statistical analysis used, were Ddo-KO values related to wild-type values at each age? Was there variation between blots as indicated from the image shown? Wild-type ratios should be included to indicate the variation. Was ANOVA used?
R. We agree with the Referee that a complete description of the procedure used for WB experiments was lacking in the original version of the manuscript. Accordingly, in the revised version the section "Subcellular fractionation and western blotting" has been drastically expanded in order to include all needed information related to quantification and statistical analysis.

Considering the very high number of experimental samples analysed in the present study and to allow for a direct comparison with electrophysisological data, we decided to focus on a careful evaluation of *Ddo*-KO and *Ddo*-WT samples at the same age more than on a longitudinal analysis of protein expression at the different time points. To this aim, for a proper comparison of *Ddo*-KO mice with *Ddo*-WT mice at the corresponding age, all *Ddo*-KO and *Ddo*-WT samples of the same age were loaded in the same gel for WB analysis. This approach was used for samples of both homogenate and triton insoluble postsynaptic fraction. Protein labeling was visualized by means of a computer-assisted imaging system (Chemidoc; Biorad). WB quantification was automatically performed using ImageLab 4.0 software (Biorad) after normalization on tubulin levels. For statistical analysis, protein levels in *Ddo*-KO mice were expressed as percentage of age-matched WT mice (run of the same gel). Data were analyzed using GraphPad Prism version 6 (GraphPad Software, La Jolla, CA, USA). Taking into account that in all cases we compared two experimental groups, data were analyzed by two-tailed Student's t-test. We omitted all *Ddo*-WT columns at 100% on the graphs considering the very high number of *Ddo*-KO columns already presented in each graph. In our opinion, this approach will lead to a better comprehension of experimental data. The symbols * and ** denote p values <0.05 and 0.01, respectively.

Increased sensitivity to phencyclidine-induced prepulse inhibition (PPI) deficits in Ddo-KO

Previous studies by this group have shown that Ddo-KO animals have reduced deficits following MK-801 compared to wild-type animals (Errico et al 2008). In this study 3 doses of PCP were used which only showed significant differences between genotypes at 6mg/kg PCP and not at 3mg/kg and 12 mg/kg. Further studies are needed to elucidate the dose-specific effects.

R. According to the Referee's suggestion in the revised version of the manuscript we reported the PCP effects on PPI disruption only at doses of 6 and 12 mg/kg. The relevance of this behavioral study is crucial for its translational meaning since it represents an *in vivo* correlate to the electrophysiological studies reported in in figs 6 and 7. On the other hand, the PPI data shown in this manuscript confirms the biphasic effect of D-aspartate during ontogenesis. Indeed, in a recently accepted work in Translational Psychiatry- Nature, we have shown that 2-month-old *Ddo*-KO mice show resilience against PCP-dependent disruption of PPI, unlike to what is shown in this manuscript in older knockout mice.

Discussion

The authors conclude that the "amplifying" glutamatergic transmission, persistent high brain levels of DAsp in Ddo mutants become adaptive at an early stage and can therefore increase NMDAR dependent synaptic plasticity and memory" However it is difficult to make these conclusions without taking into account the low levels of D-aspartate present in vivo and the relatively low affinity of D-apartate for the NMDA receptor as described by Olverman et al 1988 (also cited in the manuscript), as measured by the binding of 3H- 2-amino-5phosphononpenatonate in rat brain membranes.

R. We agree with referee's comment. In the revised manuscript we removed the mentioned discussion section.

*Verification

1. Authors report no actual or potential conflicts of interest.

2. A.U. was supported by NARSAD Independent Investigator Grant from the Brain & Behavior Research Foundation (Grant nr: 20353). F.E. was supported by grants from the Italian Ministero dell'Istruzione, dell'Università e della Ricerca (FIRB Call - Program "Futuro in Ricerca 2010" - Project nr RBFR10XCD3) and the Italian Ministero della Salute (Call Giovani Ricercatori 2009 - Project nr GR-2009-1605759).

3. All data contained in the manuscript have not been previously published and have not been submitted elsewhere and will not be submitted elsewhere while under consideration at Neurobiology of Aging.

4. All research involving animals was carried out in accordance with the European directive 86/609/EEC governing animal welfare and protection, which is acknowledged by the Italian Legislative Decree no. 116, 27 January 1992. Every effort was made to minimize suffering of the animals.

Highlights

1. D-Aspartate (D-Asp) acts as NMDAR agonist, thereby influencing NMDAR-related processes in the mammalian brain.
2. D-Aspartate Oxidase (DDO) is the only enzyme known to selectively degrade D-Asp.
2. Mice lacking *Ddo* gene display impairment in synaptic plasticity, spine morphology and memory.
3. Here we provide evidence that *Ddo* knockout mice show a substantial increase in extracellular glutamate (Glu) levels that is associated with age-dependent structural and functional alterations of the hippocampus and prefrontal cortex, as well as with a greater sensitivity to phencyclidine-induced prepulse inhibition deficits.
4. Overall, our results indicate that DDO, by strictly regulating D-Asp levels, impacts on the homeostasis of glutamatergic system and might control age-dependent NMDAR-related processes.

D-Aspartate-Oxidase influences glutamatergic system homeostasis in mammalian brain

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Abstract

We have investigated the relevance of D-Aspartate Oxidase (DDO), the only enzyme known to selectively degrade D-Asp, in modulating glutamatergic system homeostasis. Interestingly, the lack of the *Ddo* gene, by raising D-Asp content, induces a substantial increase in extracellular glutamate (Glu) levels in *Ddo* mutant brains. Consistent with an exaggerated and persistent NMDA receptor (NMDAR) stimulation, we documented in *Ddo* knockouts severe age-dependent structural and functional alterations mirrored by expression of active caspase 3 and 7 along with appearance of dystrophic microglia. In addition, elevated D-Asp content triggered in mutants alterations of NMDAR-dependent synaptic plasticity associated to reduction of hippocampal GluN1 and GluN2B subunits selectively located at synaptic sites. These effects, all of which converged on a progressive hypo-responsiveness at NMDAR sites, functionally resulted in a greater vulnerability to phencyclidine-induced prepulse inhibition deficits in mutants. In conclusion, our results indicate that DDO, by strictly regulating D-Asp levels, impacts on the homeostasis of glutamatergic system thus preventing accelerated neurodegenerative processes.

Keywords: D-Aspartate, D-Aspartate Oxidase, Glutamate, Microglia, Hippocampus, Prefrontal cortex.

1. Introduction

Of the pool of endogenous amino acids, only serine and aspartate substantially occur in free D-form in mammalian tissues (Billard, 2012,Errico, et al., 2012,Mothet and Snyder, 2012,Ota, et al., 2012,Wolosker and Mori, 2012). D-serine (D-Ser) is mainly localized in forebrain structures of the central nervous system throughout embryonic development and the postnatal phase. Compelling evidence demonstrates that D-Ser acts as an endogenous co-agonist at N-methyl-D-aspartate receptors (NMDARs) (Billard, 2012,Martineau, et al., 2006,Snyder and Kim, 2000) and facilitation of D-Ser transmission could represent a therapeutic option in psychiatric disorders, including schizophrenia (Coyle, et al., 2002). On the other hand, much less is known about the role of free D-aspartate (D-Asp) in the mammalian brain. D-Asp binds to and activates GluN2 subunits of NMDARs (Errico, et al., 2011b). In line with its pharmacological features, non-physiological elevated levels of D-Asp in knockout mice for the *D-aspartate oxidase* (*Ddo*) gene are associated with changes in NMDAR-dependent responses, including synaptic plasticity and spatial memory (Errico, et al., 2011c,Errico, et al., 2008a,Errico, et al., 2008b). Furthermore, we have previously reported that one-month oral administration of D-Asp in mice enhances NMDAR-mediated currents, basal cerebral blood volume in fronto-hippocampal areas, and increases dendritic length and spine density in prefrontal cortex (PFC) and hippocampus (Errico, et al., 2014). In addition, coherent with mouse studies, we also found that genetic variation predicting reduced DDO expression in *post-mortem* human PFC mapped to greater prefrontal grey matter and activity during working memory (Errico, et al., 2014). We also have recently documented a substantial reduction of D-Asp and NMDA in the *post-mortem* PFC and striatum of schizophrenic patients compared to healthy controls, thus suggesting a potential involvement of this molecule in schizophrenia (Errico, et al., 2013). Remarkably, D-Asp displays a peculiar temporal occurrence in the brain, i.e., levels are high between embryonic and early postnatal stages, and rapidly decrease to trace levels after birth (Dunlop, et al., 1986,Hashimoto, et al., 1993,Hashimoto, et al., 1995,Lee, et al., 1999,Neidle and Dunlop, 1990,Sakai, et al., 1998,Wolosker, et al., 2000) due to the concomitant expression of DDO, the only enzyme known to degrade bicarboxylic D-amino acids (D'Aniello, et al., 1993,Still, et al., 1949,Van Veldhoven, et al., 1991). Given the pharmacological ability of D-Asp to act as a direct NMDAR agonist and in the light of the strict control exerted by DDO on post-natal brain levels of D-Asp, we investigated the biological significance of this enzyme in regulating glutamatergic system homeostasis in the hippocampus and PFC of mice with targeted deletion of *Ddo*.

2. Materials and methods

2.1. Animals. Knockout mice for the *Ddo* gene (*Ddo*-KO) were generated and genotyped by PCR as described previously (Errico, et al., 2006). Animals were housed in a maximum of five per cage, at a constant temperature (22°±1°C) and maintained on a 12 h light/dark cycle, with food and water *ad libitum*. All research involving animals was carried out in accordance with the European directive 86/609/EEC governing animal welfare and protection, which is acknowledged by the Italian Legislative Decree no. 116, 27 January 1992. Every effort was made to minimize suffering of the animals.

2.2. In vivo microdialysis experiments. Six-month-old wild type (*Ddo*-WT) and *Ddo*-KO mice were anaesthetised with pentobarbital (50 mg/kg, i.p.), and the concentric microdialysis probes (Hutson, et al., 1985) were stereotaxically implanted in their PFC (AP: +1.5 mm, L: 0.3, and V: 3.3 mm below the *dura*) and hippocampus (AP: -3.7 mm, L: 2.4 mm from bregma and V: 4.2 mm). After a post-operative recovery period of approximately 48 hours, dialysis was commenced with artificial cerebrospinal fluid (ACSF) (pH 7.2). Animals were perfused at a rate of 1.2 µl/min using a Harvard Apparatus infusion pump (mod. 22) and samples were collected every 20 min. Dialysates were analysed for Glu content using a high performance liquid chromatography (HPLC) coupled with fluorimetric detection method. The mean concentration of Glu in the dialysates of first five samples represents the basal release, and results are expressed as a concentration of pmol/10 µl. Microdialysis experiments in the PFC of C57BL/6J after acute injection of D-Asp were performed as previously described (Mabrouk, et al., 2010). Samples were collected every 20 min, starting 6 hour after the onset of probe perfusion. At least 3 stable values were collected before administering D-Asp (500 mg/kg, i.p.). Glu levels were measured by HPLC coupled with fluorimetric detection as previously described (Mabrouk, et al., 2010, Marti, et al., 2007).

2.3. Synaptosomal experiments. Cortical synaptosomes from C57BL/6J mice were prepared as previously described (Marti, et al., 2003, Mela, et al., 2004). The synaptosomal pellet was re-suspended in oxygenated (95% O_2, 5% CO_2) Krebs solution (mM: NaCl 118.5, KCl 4.7, $CaCl_2$ 1.2, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 10). One-milliliter aliquot of the suspension (~0.35 mg protein) was slowly injected into nylon syringe filters (outer diameter 13 mm, 0.45 µM pore size, internal volume of about 100 µl; Teknokroma, Barcelona, Spain) connected to a peristaltic pump. Filters were maintained at 36.5°C in a thermostatic bath and superfused at a flow rate of 0.4 ml/min with a pre-oxygenated Krebs solution containing the excitatory amino acid transporter inhibitor DL-*threo*-β-benzyloxyaspartic acid (DL-TBOA). Sample collection (every 3 min) was initiated after a 20 min period of filter washout. The effects of D-Asp and NMDA were evaluated

on 15 mM K^+-stimulated (90 sec pulse) neurotransmitter outflow. Compounds were added to the perfusion medium 3 min before K^+ and for additional 3 min, whereas MK-801 was perfused 3 min before the addition of D-Asp or NMDA.

2.4. Immunofluorescence. Ddo-WT and *Ddo*-KO mice at 0.5, 3 and 6 months of age (n=6 per age and genotype group) were deeply anesthetized and transcardially perfused with a saline solution followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Morphological investigation of the glutamatergic inputs was performed by immunohistochemical study of VGluT1/synaptophysin co-expression on transverse serial sections of brain (10-μm thick) incubated with a mixture of polyclonal primary antibodies (rabbit anti-vesicular glutamate transporter 1, Synaptic System; and guinea pig anti-synaptophysin, Cell Signalling) revealed by appropriate mixture secondary antibodies (AlexaFluorTM). The distribution of glutamatergic inputs was analyzed with a laser-scanning confocal microscope (LSM510 Meta, Zeiss; n=20 serial sections for each sample per group) according to the conventional method of synaptological analysis as previously described (Cristino, et al., 2013). Immunohistochemical study of caspase 3 and 7 reactivity in hippocampus and prefrontal cortex was performed by incubation of sections with goat anti-active caspase 3 or mouse anti-active caspase 7 (Santa Cruz Biothecnology, Santa Cruz CA, USA) revealed by specific Alexa-488 secondary donkey anti-IgGs antibody (Invitrogen Life Technology, Paisley, UK) and counterstained with DAPI (4',6-diamidino-2-phenylindole). Morphological investigation of microglia and astrocytes was performed by incubation of sections with rabbit anti-Iba-1 primary antibody (anti-ionized calcium binding adapter molecule 1; Wako Chemicals), rabbit anti-*glial fibrillary acidic protein* (GFAP) and revealed by appropriate secondary antibody (AlexaFluorTM). Immunoreactivity for caspase 3, caspase 7, Iba-1 or GFAP was analyzed by DMI6000 microscope equipped with appropriate filters and deconvolution MetaMorph LAS AF 2.2.0 software (Leica, Germany). The images were acquired using digital camera DFC 340FX (Leica) connected to the microscope. Iba-1 and GFAP-positive cells were identified as resting (with small somata bearing long, thin and ramified processes), activated microglia and astrocytes (with hypertrophy together with retraction of processes to a length shorter than the diameter of the somata) or dystrophic microglia (no dystrophic astrocytes were detected). Dystrophic microglia was recognized by debris consisting of several cells displaying fragmented processes and an irregularly shaped cell body as previously demonstrated in humans (Streit, et al., 2009). Quantitative analysis was performed by counting, for each phenotype, the bisbenzimide-counterstained cells with nucleus on the focal plane within a box measuring $2x10^4$ μm^2 in the lateral, central and medial areas of the CA1 hippocampus or in PFC.

2.5. Electron microscopy. Ddo-WT and *Ddo*-KO mice at 0.5 and 6 months of age (n=3 per age and

genotype group) were deeply anesthetized and perfused transcardially with PBS (pH 7.4) followed by a fixative containing 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Ultrathin sections (60-nm thick) containing the CA1 hippocampal field were collected serially on Formovar-coated, single- or multiple-slot (50-mesh) grids, stained with 0.65% lead citrate and examined with the Zeiss LEO 912AB transmission electron microscope. In these blinded experiments, synapses were identified based on postsynaptic density, and at least three vesicles in the presynaptic element. Excitatory synapses were identified by their asymmetrical morphology, and inhibitory synapses ones by their symmetrical morphology. We used the physical dissector method to count synapse and their distribution according to the conventional ultrastructural synaptological analysis described elsewhere (Kleim, et al., 1996).

2.6. Electrophysiology. Vibratome-cut parasagittal slices (400-μm thick) were prepared, incubated for 1 hour and then transferred to a recording chamber submerged in continuously flowing artificial cerebrospinal fluid (ACSF, 30°C, 2–3 ml/min) gassed with 95% O_2 and 5% CO_2. The composition of the control solution was (in mM): 126 NaCl, 2.5 KCl, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 2.4 $CaCl_2$, 11 glucose, 25 $NaHCO_3$. Field excitatory post-synaptic potentials (fEPSPs) were evoked by stimulating the Schaffer collateral/commissural pathway with a bipolar NiCr insulated wire electrode placed in the *stratum radiatum* of the CA1 region. Extracellular recordings were made in the *stratum radiatum* using 3 M NaCl-filled glass microelectrodes with a resistance of 2–3 MΩ. A stimulation intensity that evoked 30% of the maximal fEPSP response was used. LTP was evoked with theta-burst stimulation (TBS: 4 trains of 5 pulses at a frequency of 100 Hz, with an inter-train interval of 200 ms). Whole cell patch-clamp recordings were obtained as previously described (Nistico, et al., 2013). The AMPAR and NMDAR current-voltage relationship and the AMPAR/NMDAR ratio were obtained from excitatory post-synaptic currents (EPSCs) elicited at 0.033 Hz with a glass pipette filled with ACSF, close to the dendritic region of the recorded neuron. AMPAR peak currents were estimated in the presence of 100 μM picrotoxin plus 100 μM APV and normalized to the EPSC at -60 mV. The NMDAR currents were measured with peak amplitudes in the presence of 100 μM picrotoxin and 10 μM NBQX and normalized to the EPSC at +60 mV. AMPAR/NMDAR ratios were first recorded at +40 mV in the presence of 100 μM picrotoxin plus 100 μM APV, followed by APV washout (15 min) and addition of 10 μM NBQX to reveal NMDAR-EPSCs. *n* refers to the total number of slices used or neurons recorded. Significance of data was determined using the two-tailed unpaired Student's *t*-test or one-way ANOVA, as appropriate.

2.7. Subcellular fractionation and western blotting. Subcellular fractionation of the hippocampus

and cortex from *Ddo*-WT and *Ddo*-KO mice was performed as previously described (Gardoni, et al., 2006). The protein content of the samples was quantified with the Bio-Rad (Hercules, CA, USA) protein assay. After measuring protein concentration, all samples have been standardized at 1 μg/μl concentration. Protein samples (10 μg/sample loaded in each lane) were separated onto 7-9% acrylamide/bisacrylamide gel, transferred to a nitrocellulose membrane and probed with the appropriate primary and HRP-conjugated secondary antibodies. Samples from *Ddo*-WT and age-matched *Ddo*-KO mice were loaded in the same gel. Western Blot analysis was performed on the homogenate and triton insoluble postsynaptic fraction (TIF) using antibodies raised against the GluN1, GluN2A and GluN2B subunits of the NMDAR, GluA1 and GluA2 subunits of the AMPAR and tubulin. Labeling was visualized by means of a computer-assisted imaging system (Chemidoc; Biorad). WB quantification was performed using ImageLab 4.0 software (Biorad) after normalization on tubulin levels. For statistical analysis, protein levels in *Ddo*-KO mice were expressed as percentage of age-matched *Ddo*-WT mice. Data were analyzed using GraphPad Prism version 6 (GraphPad Software, La Jolla, CA, USA). Data were analyzed by Student's t-test by comparing KO with age-matched WT mice. The symbols * and ** denote p values <0.05 and 0.01, respectively.

2.8. Antibodies. We used the following antibodies: polyclonal GluN2A antibody (Sigma-Aldrich, St. Louis, MO); monoclonal GluN1 antibody (Life Technologies Carlsbad, CA); monoclonal GluN2B, GluA1 and GluA2 antibodies (NeuroMab, Davis, CA); and monoclonal tubulin antibody (Sigma-Aldrich).

2.9. Prepulse inhibition of the startle reflex under PCP treatment. Phencyclidine hydrochloride (PCP) (Sigma-Aldrich) was dissolved in saline (0.9% NaCl) and administered intraperitoneally (i.p.) in a volume of 10 ml/kg. Prepulse inhibition (PPI) of the acoustic startle response was measured using the SR-Lab System (San Diego Instruments, San Diego, CA, USA). The protocol is described elsewhere (Errico, et al., 2008b). Briefly, *Ddo*-WT and *Ddo*-KO mice of 6 months of age were randomly assigned to the following groups: 6 mg/kg PCP, 12 mg/kg PCP or Veh (balanced for genotype and startle chamber assignment), and placed in the Plexiglas cylinder of the startle chamber 10 min after i.p. injection. We tested 70, 74, 78 and 82 dB prepulse sounds on a background noise level of 65 dB. Prepulse inhibition was used as dependent variable and analyzed within genotypes by two-way (treatment x prepulse sound levels) ANOVA with repeated measures. Statistical analyses were performed with StatView software (version 5.0.1.0; SAS Institute).

3. Results

3.1. D-Asp modulates Glu release in mouse brain and in synaptosomal preparation

We previously reported that D-Asp levels in the cortex and hippocampus homogenates were 15-20-fold higher in *Ddo*-KO than in *Ddo*-WT littermates (Errico, et al., 2012). Here we investigated the consequences of elevated D-Asp content on basal extracellular Glu levels in the brain of *Ddo*-KO mice. *In vivo* microdialysis performed in the hippocampus of freely moving mice revealed that Glu levels were significantly higher in 6-month-old mutants than in age-matched controls [$F_{(1,12)}$=14.87; $p<0.01$] (Fig. 1A). To investigate the neurobiological basis underlying Glu system dysregulation found in mutants, we evaluated whether D-Asp application triggered Glu release from cortical synaptosomal preparations obtained from C57BL/6J mice. We found that Glu levels in cortical synaptosomes exposed to the uptake inhibitor DL-TBOA (10 μM) were 20.3±1.7 nM (n=59). A 90-sec pulse with K^+ transiently increased Glu levels, which peaked (~60% above basal) in the stimulation sample and returned to baseline in the subsequent sample (not shown). Remarkably, D-Asp, ineffective at 1 μM, potentiated the K+-induced Glu overflow (10.8±1.1 pmol/mg prot/min) by more than 3-fold, when used at 10 μM (34.5±7.7 pmol/mg prot/min) [$F_{(3,26)}$=5.01; p=0.0071]. Interestingly, NMDA and D-Asp produced the same Glu efflux at 10 μM (Fig 1B). However, neither D-Asp (10 μM) nor NMDA (10 μM) significantly potentiated the K^+-stimulated Glu overflow in the presence of MK-801 (1 μM) (NMDA+MK-801 9.3±2.5 pmol/mg prot/min, t=0.65, df=16, p=0.52; D-Asp+MK-801 12.2±2.6 pmol/mg prot/min, t=0.57, df=14; p=0.57), which indicates that presynaptic NMDARs mediate this effect.

Next, to verify *in vivo* whether acute administration of D-Asp modulates Glu neurotransmission in control brains, we performed microdialysis in freely moving C57BL/6J mice injected with 500 mg/kg D-Asp. In line with *in vitro* experiments, we documented that acute D-Asp injection triggered a significant Glu release in the PFC of mice when compared with saline injected C57BL/6J animals [$F_{(1,20)}$=5.77; $p<0.05$] (Fig. 1C).

3.2. Age-dependent reduction of excitatory synaptic input to the pyramidal neurons of the hippocampal CA1 field and of the IL/PL cortex in *Ddo*-KO brain

In the light of the consistent alterations of glutamatergic neurotransmission observed in the mutant hippocampus, we next investigated the neuroanatomical features of the glutamatergic system in the CA1 hippocampal field of *Ddo*-KO mice at the age of 0.5, 3 and 6 months. To this aim, we evaluated VGluT1/synaptophysin expression, which identifies excitatory presynaptic nerve terminals (Cristino, et al., 2013). Interestingly, in CA1 field at 0.5 months of age, we reported a consistent increase of vGluT1 and synaptophysin in *Ddo*-KO brains (Fig. 2; compare A1 vs. B1).

Conversely, a robust age-dependent reduction of VGluT1 and synaptophysin were found in the CA1 field of *Ddo*-KO mice, compared to their respective littermates, starting at 3 months of age (Fig. 2; compare C1 vs. D1, and E1 vs. F1 in *stratum oriens*, s.o., *radiatum*, s.r., and *pyramidale*, s.p.). Interestingly, in mutants a similar effect also occurred in *laminae* II/III of the infralimbic/prelimbic (IL/PL) cortex (Fig. 2G-L).

To further determine the effect of D-Asp dysregulation on the synaptic arrangement of inhibitory or excitatory inputs to hippocampal CA1 and IL/PL pyramidal neurons, we performed unbiased electron microscopic analysis in 0.5- and 6-month-old *Ddo*-KO animals in which the immunohistochemical differences were more pronounced. Analysis of variance (ANOVA) between ages did not reveal any significant difference in the number of symmetrical (i.e. inhibitory) or asymmetrical (i.e. excitatory) synapses in CA1 (Fig. 2M,O,Q) [$F_{(5,39)}$=1.8; p=0.14] and IL/PL (Fig. 2Q) [$F_{(5,65)}$=1.9; p=0.15] of *Ddo*-WT mice. On the contrary, there was a robust loss of asymmetrical excitatory synapses at both axosomatic and axodendritic compartments in 6-month-old *Ddo*-KO mice, compared to 0.5-month-old *Ddo*-KO mice in both CA1 [$F_{(1,105)}$=16.833, p<0.0001] and IL/PL [$F_{(1,108)}$=17.593, p<0.0001], whereas the number of symmetrical excitatory synapses did not differ from values obtained in *Ddo*-WT mice (Fig. 2N, P). Interestingly, we found an opposite trend in asymmetrical synapse changes between *Ddo*-WT and *Ddo*-KO mice. Indeed, the number of hippocampal asymmetrical synapses was significantly higher in 0.5-month-old *Ddo*-KO mice than in age-matched controls [$F_{(5,48)}$=3.8; p<0.05], (Fig. 2Q). Conversely, at 6 months of age the number of excitatory asymmetrical synapses was significantly lower than in aged-matched littermates (Fig. 2Q) [$F_{(1,48)}$=5.568, p<0.05]. Taken together, these neuroanatomical data provide direct evidence that persistent higher brain levels of D-Asp in mutants severely affect the morphology of glutamatergic system.

3.3. Appearance of dystrophic microglia and reactive astrocytes in the brain of *Ddo*-KO mice

Given the dysfunctional Glu system in *Ddo* mutant brains, and based on the well-established relationship between Glu and neuroinflammation (Vesce, et al., 2007), we investigated whether distribution and morphology of microglia and astrocytes were altered in the PFC and hippocampus of *Ddo* knockouts.

In the PFC, the total number of microglia and the hypertrophic activated phenotype did not differ between *Ddo*-WT and *Ddo*-KO mice at the three ages (0.5, 3 and 6 months) examined (total cell number $F_{(5,54)}$=1.08 p=0.34; hypertrophic cell number $F_{(5,54)}$=1.43, p=0.248) (Fig. 3A,B). However, at 6 months of age, *Ddo*-KO brains showed the occurrence of a peculiar dystrophic-like microglial phenotype, not found in age-matched controls. The specificity of this microglia morphology is

characterized by fragmented cytoplasm (cytorrhexis) previously reported in degenerating human neuronal brain structures (Streit, et al., 2009) (Fig. 3A,B). On the other hand, in the CA1 region of mutant hippocampi at 0.5 months of age we found a higher number of total Iba-1-positive microglia together with a greater number of hypertrophic phenotypes (total cell number $F_{(5,54)}$=17.93, p<0.0001; hypertrophic cell number $F_{(5,54)}$=38.04, p<0.0001) (Fig. 3C,D). These results were not observed at the other ages analysed (Fig. 3C,D). Similarly to PFC, also in the hippocampus of mutants we found the occurrence of dystrophic-like microglia at 6-months of age (Fig. 3C,D).

We then analysed the distribution and morphology of astrocytes. GFAP analysis revealed that the number of both total and reactive astrocytes was significantly higher in the PFC of 6-month-old *Ddo*-KO mice, compared to their age-matched controls (total cell number $F_{(1,18)}$=22.83 p<0.001; reactive cell number $F_{(1,18)}$=20.48, p<0.001. Nevertheless, no significant changes between genotypes were observed in this brain region at 0.5 months (total cell number $F_{(1,18)}$= 2.07 p=0.155; reactive cell number $F_{(1,18)}$=2.29, p=0.129) (Fig. 4A,B). On the contrary, in the hippocampus, we found an increased number of both total and reactive astrocytes in 0.5-month-old *Ddo*-KO mice, compared to their age-matched controls (total cell number $F_{(1,18)}$=10.04, p<0.01; reactive cell number $F_{(1,18)}$=53.96, p<0.0001) (Fig. 4C,D). Moreover, in 6-month-old *Ddo*-KO mice, the total number of astrocytes was significantly higher, compared to their age-matched controls, while the number of hypertrophic (reactive) astrocytes did not significantly change (total cell number $F_{(1,18)}$=24.36, p<0.001; reactive cell number $F_{(1,18)}$=3.12 p=0.057) (Fig. 4C,D).

3.4. Higher expression of active caspase 3 and 7 in the hippocampus and PFC of *Ddo*-KO mice

To investigate whether the progressive morphological alterations found in the brain of *Ddo*-KO mice may depend on the activation of excitotoxic and apoptotic events, we analyzed the expression of active caspase 3 and caspase 7 (Lakhani, et al., 2006). Activation of caspases plays a pivotal role in neuronal apoptosis and is considered the terminal event preceding cell death (Shalini, et al., 2014). Consistent with the detrimental effect of cerebral D-Asp dysregulation in mutants, we found a significant increase of immunoreactivity for activated caspase 3 (Fig.5 A-L_1) and caspase 7 (Fig.5 M-X_1) in the nuclei of pyramidal neurons of hippocampal CA1 subfield and in *laminae* II/III of IL/PL PFC area of 6-month-old *Ddo*-KO, compared to age-matched controls. Noteworthy, an early onset for activated caspases 3 and 7 immunoreactivity was already found both in CA1 (Fig. 5, C-D_1, O-P_1) and PFC (Fig. 5, I-J_1, U-V_1) area of 3-month-old *Ddo*-KO but not in control mice. Notably, at 0.5 months of age, we did not find any genotype difference in the levels of activated caspases 3 and 7, in both brain regions analyzed (Fig. 5, A-B_1, M-N_1, CA1 subfield) (Fig. 5, G-H_1, S-T_1, IL-PL cortex).

Overall, the expression of activated caspases 3 and 7 suggests the existence of consistent remodeling neuronal processes and excitotoxic events in the hippocampus and PFC regions of *Ddo*-KO mice.

3.5. Age-dependent changes in LTP and synaptic NMDAR subunit composition in the hippocampus of *Ddo*-KO mice

Based on the established role played by Glu on synaptic plasticity and considering the severe alterations reported in the Glu system of *Ddo* mutants, here we analysed the NMDAR-dependent long-term potentiation (LTP) in the CA1 region of *Ddo*-KO mice at different ages (Fig. 6A). At 0.5 months, the magnitude of LTP did not differ between *Ddo*-KO and *Ddo*-WT mice ($p>0.05$). Conversely, at 3 months, LTP was significantly higher in mutants than in control slices ($p<0.001$), whereas it progressively decreased in *Ddo*-KO mice between 6 and 12 months of age. In particular, no statistical differences appeared between 6-month-old *Ddo*-KO and age-matched control mice ($p>0.05$), whereas LTP was dramatically impaired in 12-month-old *Ddo*-KO animals, compared to their relative controls ($p<0.001$).

To explore the possibility that alterations in synaptic plasticity might be correlated to molecular modifications and distribution of the ionotropic glutamate receptors at synapses, we measured protein levels of NMDAR and AMPAR subunits in postsynaptic fractions purified from the hippocampus and cortex of *Ddo*-KO and *Ddo*-WT mice. Western blot analyses indicated that levels of the NMDAR subunits did not differ in hippocampal synapses between 0.5-month-old *Ddo*-KO mice and their age-matched controls ($p>0.05$, Student's *t*-test) (Fig. 6B). However, a significant progressive decrease of the synaptic NMDARs, mainly composed by GluN1/GluN2B subunits was observed at 1, 3, 6 and, more robustly, at 12 months in *Ddo*-KO mice compared to their relative controls ($p< 0.05$ Student's *t*-test) (Fig. 6B). This effect was specific for NMDAR subunits because there was only a trend of reduction of synaptic levels of AMPAR subunits in *Ddo*-KO mice (Fig. 6C). Notably, the AMPAR and NMDAR subunits at cortical synapses did not differ between mutant and *Ddo*-WT mice (data not shown). Finally, all changes observed occurred at the synaptic locations of the NMDAR subunits and not in the soma. In fact, western blotting performed in hippocampal and cortical total cell homogenates did not show any significant reductions in the levels of any of the ionotropic glutamate receptor subunits tested (data not shown).

3.6. Age-related changes in the AMPAR/NMDAR ratio in *Ddo* mutant brains

To test if LTP changes in *Ddo*-KO mice could also depend on altered NMDAR or AMPAR channel properties, we carried out whole-cell voltage-clamp recordings from CA1 pyramidal neurons. As

shown in Fig. 7, no differences were evident in current–voltage (I-V) relationship neither in the excitatory postsynaptic currents mediated by AMPAR nor by NMDAR in 0.5-, 3- and 6-month-old *Ddo*-KO mice compared to age-matched controls ($p>0.05$ Student's *t*-test) (Fig. 7A,B,C). Conversely, the AMPAR/NMDAR ratio was decreased in 0.5- and 3-month-old *Ddo*-KO, compared to age-matched *Ddo*-WT mice ($p<0.001$ Student's *t*-test) (Fig. 7A,B), whereas no difference was found in 6-month-old *Ddo*-KO vs. age-matched control mice ($p>0.05$ Student's *t*-test) (Fig. 7C). Importantly, an age-related analysis in *Ddo* mutants reveals a progressive increase in the AMPAR/NMDAR ratio ($p<0.001$ one-way ANOVA) (Fig. 7D), indicating that changes in NMDAR or AMPAR channel properties might represent adaptive responses following persistently elevated D-Asp brain levels.

3.7. Increased vulnerability to phencyclidine-induced prepulse inhibition deficits in 6-month-old *Ddo*-KO mice

Sensorimotor gating, measured by PPI, relies on intact NMDAR transmission (Duncan, et al., 2004). We recently demonstrated that 2-3-month-old *Ddo*-KO mutants, characterized by higher NMDAR-transmission (Errico, et al., 2011a,Errico, et al., 2014,Errico, et al., 2011b,Errico, et al., 2011c,Errico, et al., 2008a,Errico, et al., 2008b), exhibit a significant resiliency against PPI deficit elicited by PCP (Errico, et al., 2015, in press). In keeping with this, here we performed PPI experiment after treatment with PCP in 6-month-old animals (Fig. 8), an age at which mutants already show overt morphological alterations in glutamatergic system.

Interestingly, at the dose of 6 mg/kg, we found that PCP did not affect PPI in *Ddo*-WT mice [treatment effect: *Ddo*-WT, $F_{(1,48)}=0.002$, $p=0.9658$], while it significantly affected startle reflex inhibition in *Ddo*-KO mice [treatment effect: *Ddo*-KO, $F_{(1,48)}=5.568$, $p<0.05$] compared to their relative vehicle-treated groups (Fig. 8A). Conversely, a higher dose of PCP (12 mg/kg) induced a substantial disruption of PPI in both genotypes [*Ddo*-WT, $F_{(1,54)}=10.684$, $p=0.01$; *Ddo*-KO, $F_{(1,54)}=9.010$, $p=0.01$] (Fig. 8B). These *in vivo* data indicate enhanced sensitivity to PPI deficits induced by PCP in *Ddo*-KO mice, and provide a behavioral correlate for the robust NMDAR-hypofunctionality state found in these animals from 6 months of age.

4. Discussion

Here we document that DDO plays a role in the modulation of glutamatergic system homeostasis in the mammalian brain. Indeed, deregulation of D-Asp brain levels, through genetic-deletion of the *Ddo* gene, triggers a consistent change in extracellular Glu levels. The ability of D-Asp to enhance

Glu release is confirmed either following its acute injection in freely moving mice or by its direct application in the synaptosome preparation. Synaptosomes in superfusion are an ideal preparation for studying basic mechanisms of neurosecretion and its presynaptic modulation, since a low K^+ concentration evokes exocytotic neurotransmitter release (Marti, et al., 2003). The superfusion conditions and the presence of the transporter inhibitor DL-TBOA in the medium exclude reversal of Glu transport. Under these conditions, D-Asp facilitated K^+-evoked Glu overflow. Since this effect was replicated by NMDA and blocked by MK-801, presynaptic NMDARs appear to be involved in D-Asp action. On the other hand, the ability of D-Asp to elicit Glu release appears to be brain region-specific because systemic injection of D-Asp did not result in a simultaneous increase of Glu release in the striatum of C57BL/6J mice (data not shown). Consistent with the lack of a stimulatory effect of D-Asp on striatal Glu release, we have previously found that NMDA was unable to stimulate Glu release from striatal synaptosomes (Marti, et al., 2002). This is likely due to the absence of NMDA autoreceptors in this brain area, since NMDA was able to stimulate GABA release from the same synaptosomal preparation (Marti, et al., 2002). Future studies are mandatory to elucidate the mechanism by which D-Asp crosses the blood brain barrier influencing Glu release in a region-specific manner.

In agreement with neurochemical observations, we documented that persistently elevated D-Asp and Glu levels in *Ddo*-KO brains are associated with progressive morphological alterations in hippocampal and cortical glutamatergic terminals, as detected by immunofluorescence microscopy. Moreover, 6-month-old *Ddo*-KO mice also show a significant reduction of excitatory glutamatergic inputs to pyramidal neurons of hippocampal CA1 subfield and *laminae* II/III of the IL/PL cortex, paralleled by a consistent expression of active caspase 3 and caspase 7. Altogether, the progressive dysfunctional events observed in the brain of *Ddo*-KO animals indicate that DDO enzyme plays a neuroprotective role in preventing early neurodegenerative processes. We argue that the emergence of such detrimental events could be a direct consequence of the altered Glu release occurring in *Ddo*-KO mice in response to deregulated high brain levels of D-Asp.

Mounting evidence suggests a link between Glu dysfunction and neuroinflammatory processes, which in turn might contribute to the pathogenic mechanisms of neurological (Ringheim and Szczepanik, 2006,Vesce, et al., 2007) and neuropsychiatric conditions such as depression (Dantzer and Walker, 2014,Kreisel, et al., 2014) and schizophrenia (Muller, 2008). In agreement with the severe alteration in the glutamatergic system observed in adult mutants, we found increased number of reactive astrocytes in the PFC of 6-month-old *Ddo*-KO mice. Interestingly, at the same age, we also revealed the appearance of a dystrophic microglia phenotype in both the hippocampus and PFC of mutants. Together with the increased active caspase 3 and 7 expression, these morphological

features suggest the existence of inflammatory processes in the brain lacking functional DDO.

It has been reported that deficient neuron-microglia signalling (Zhang, et al., 2014) and caspase 3 activation (Jo, et al., 2011) affect synaptic plasticity. In this regard, we found that *Ddo*-KO mice exhibit a progressive decay of LTP from 6 months of age onwards when microglia display an overt morphological abnormality and active caspase 3 and 7 are expressed throughout the hippocampus. On the other hand, age-related LTP deficit seen in *Ddo* mutant hippocampi could be further explained by and selective reduction of synaptic GluN1- and GluN2B-containing NMDARs. We believe that remodelling of the hippocampal glutamatergic synapses might represent an adaptive mechanism to counteract non-physiological overstimulation of NMDARs in these mutants. Accordingly, it is conceivable that mechanisms already described in neurodegenerative conditions (Mellone and Gardoni, 2013,Sanz-Clemente, et al., 2013), i.e. increased receptor endocytosis or a lateral movement to extra-synaptic sites, could account for the above-mentioned molecular event. Consistent with this concept and in agreement with our results, specific defects in the synaptic localization of NMDARs have been reported in age-related disorders in both animal models and humans (Paoletti, et al., 2013,Rosenzweig and Barnes, 2003). The progressive reduction of GluN1/GluN2B subunits in mutant hippocampi could be related to the early cognitive decay observed in aged *Ddo*-KO mice when tested in context-dependent paradigms (Errico, et al., 2011c). On the other hand, patch-clamp experiments suggest no differences in the AMPAR and NMDAR current-voltage relationship between *Ddo*-KO and *Ddo*-WT mice at the three age-stages tested. On the contrary, and in line with our biochemical experiments, the AMPAR/NMDAR ratio was progressively increased in *Ddo*-KO mice between 0.5 and 6 months of age. Since both NMDARs and AMPARs are important in the generation of CA1-LTP (Malinow and Malenka, 2002), changes in the AMPAR/NMDAR ratio might explain the synaptic plasticity alterations in *Ddo* knockouts. Accordingly, LTP was enhanced in *Ddo*-KO mice at an early stage (3 months), when the AMPAR/NMDAR ratio was reduced. Conversely, it progressively decays from 6 months of age onwards in parallel with the increase in the AMPAR/NMDAR ratio.

Our previous and present data suggest that persistent non-physiological higher levels of D-Asp in *Ddo*-KO brains may promote NMDAR-dependent synaptic transmission, neuroplasticity (Errico, et al., 2014,Errico, et al., 2011b,Errico, et al., 2011c,Errico, et al., 2008a) and memory (Errico, et al., 2011b,Errico, et al., 2011c,Errico, et al., 2008b), as well as resilience against MK801- and PCP-induced schizophrenia-related behaviours in young-adult mice (Errico, et al., 2008b), Errico, et al., 2015 in press). On the contrary, non-physiological prolonged elevation of D-Asp from 6 months of age onwards is associated with altered glutamatergic transmission and severe structural defects which, over time, renders the brain refractory to NMDAR-mediated processes, producing

precocious brain aging features (Errico, et al., 2011c).

In addition, our behavioural data showing a greater susceptibility to PCP-induced sensorimotor gating alterations in 6-month-old mutants are in contrast to those found in 2/3-month-old mutants (Errico, et al., 2015 in press). The apparent discrepancy in the PPI response following PCP administration might be explained by the postulated age-sensitive responsiveness of NMDAR in *Ddo*-KO brains. In conclusion, the present work documents an unexpected role for DDO in modulating Glu system homeostasis, thereby preventing precocious age-related deterioration processes.

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Figure Legends

Figure 1. Lack of *Ddo* gene or acute injection of D-Asp induces a significant increase in glutamate levels in *Ddo*-KO compared to *Ddo*-WT mice A) *Ddo*-KO mice showed significantly higher glutamate levels (8.98±1.27 pmol/10 µl, n=7) than *Ddo*-WT mice (3.13±0.83 pmol/10 µl; n=7) in the hippocampus. B) D-Asp and NMDA potentiated the 15 mM K^+-evoked glutamate overflow in cortical synaptosomes in superfusion. C) Systemic administration of D-Asp in naïve mice significantly increased glutamate release in IL/PL cortex Glu concentrations (Vehicle: 0.44±0.33, n=12; D-Asp: 0.76±0.05, n=10). Values are expressed as mean±SEM of *n* determinations; **p<0.01 and ***p<0.001 (two-way ANOVA followed by the Bonferroni post-hoc test).

Figure 2. Age-related synaptic impairment of excitatory inputs to the hippocampal CA1 region and cortical (IL/PL) pyramidal neurons in *Ddo*-KO mice. A-L) Immunofluorescence showing VGluT1 (red) and synaptophysin (green) immunoreactivity in the hippocampal CA1 field (A-F_1) and in *laminae* II/III of the infralimbic cortex (G-L) in *Ddo*-WT and *Ddo*-KO mice. Note the high density of synaptophysin labelling in the *stratum oriens* (s.o.), *pyramidale* (s.p.) and *radiatum* (s.r.) of 0.5 months *Ddo*-KO mice vs. age-matched *Ddo*-WT, and its massive reduction, mainly affecting excitatory inputs, during ontogenesis Note the co-localization of VGluT1 and synaptophysin in the axonal terminals surrounding the unlabeled soma of the pyramidal neurons in the hippocampus (s.p.) and IL/PL cortex (G-L). A_1-D_1, F_1). High magnification of the boxed area depicted in the respective CA1 fields. M-P): Representative electron micrographs showing the synaptic axo-dendritic arrangement in the CA1 *stratum pyramidale* (s.p.) of *Ddo*-WT (M,O) and *Ddo*-KO (N,P) mice. Note the high density of asymmetrical synapses (blue arrows, i.e. putative excitatory) versus symmetrical synapses (dark arrows, i.e. putative inhibitory) in 0.5-month-old *Ddo*-KO vs. age-matched *Ddo*-WT mice, and its reduction during ontogenesis. Q) Bar graph of density of symmetrical or asymmetrical synapses in CA1 (s.p.) and IL/PL cortex *laminae* II/III of 0.5- and 6-month-old *Ddo*-WT and *Ddo*-KO mice determined by immunoelectron microscopy. At, axon terminal; sp, dendritic spine (si potrebbe confondere con s.p., strato piramidale riportato nei pannelli in alto). Scale bar: 100 µm (A-L); 50 µm A_1-F_1; 1 µm (M-P). Data are presented as mean±SEM by comparing symmetrical vs. asymmetrical synapses of *Ddo*-WT and *Ddo*-KO mice both at 0.5 and 6 months. *p<0.05; ***p<0.0001. ANOVA followed by Bonferroni *post hoc* test were used for statistical analysis.

Figure 3. Morphological analysis of microglia in the medial prefrontal cortex and hippocampus of 0.5-, 3- and 6-month-old *Ddo*-WT and *Ddo*-KO mice. A) Immunofluorescence of Iba-1-positive

microglial cells in the medial prefrontal cortex (mPFC) of 0.5-, 3- and 6-month-old *Ddo*-WT (upper panel) and *Ddo*-KO (lower panel) mice. Insets show the region of interest at higher magnification. Single cells on the right of the panel are a representative example of the microglial morphology in the 0.5- and 6-month-old *Ddo*-WT (upper) and *Ddo*-KO (lower) mice. No significant changes were revealed in the 3-month-old *Ddo*-WT and *Ddo*-KO mice. Note the appearance of dystrophic and fragmented microglia in the 6-month-old *Ddo*-KO mPFC, compared with the respective *Ddo*-WT cells. B) Quantitative analysis of the total, hypertrophic and dystrophic-like cells in the mPFC of 0.5-, 3- and 6-month-old *Ddo*-WT and *Ddo*-KO mice. C) Immunofluorescence for Iba-1-positive microglial cells in the hippocampus of the 0.5-, 3- and 6-month-old *Ddo*-WT (upper panel) and *Ddo*-KO (lower panel) mice. Insets show the region of interest at a higher magnification. Single cells on the right of the panel are a representative example of the different microglial morphology in the 0.5- and 6-month-old *Ddo*-WT (upper) and *Ddo*-KO (lower) mice. Note the hypertrophic microglia in 0.5-month-old *Ddo*-KO mice and the dystrophic-like morphology in 6-month-old *Ddo*-KO mice compared to respective age-matched *Ddo*-WT mice. D) Quantitative analysis of the total, hypertrophic and dystrophic-like cells in the hippocampus (CA1 subfield) of 0.5-, 3- and 6-month-old *Ddo*-WT and *Ddo*-KO mice. Data are presented as mean±SEM. ANOVA followed by Tukey *post hoc* test was used for statistical analysis. Four animals were used for each experimental group. Scale bar 100, 50 and 25 μm for panoramic, inset and single cell image, respectively.

Figure 4. Morphological analysis of astrocytes in the medial prefrontal cortex and hippocampus of 0.5 and 6-month-old *Ddo*-WT and *Ddo*-KO mice. A) Immunofluorescence for GFAP-positive astrocytes in the medial prefrontal cortex (mPFC) of 0.5 and 6-month-old *Ddo*-WT (upper panel) and *Ddo*-KO (lower panel) mice. Insets show the region of interest at higher magnification. B) Quantitative analysis of the total and hypertrophic reactive astrocytes in the mPFC of 0.5 and 6-month-old *Ddo*-WT and *Ddo*-KO mice (hypertrophic cells 2±0.68 vs. no cells; total cells 8.82±1.16 vs 4.13±0.22 in 6-month-old *Ddo*-KO and *Ddo*-WT, respectively). C) Immunofluorescence for GFAP-positive astrocytes in the hippocampus of 0.5- and 6-month-old *Ddo*-WT (upper panel) and *Ddo*-KO (lower panel) mice. Insets show the region of interest at a higher magnification. D) Quantitative analysis of the total and hypertrophic reactive astrocytes in the hippocampus (CA1 subfield) of 0.5- and 6-month-old *Ddo*-WT and *Ddo*-KO mice (hypertrophic cells 4±0.02 vs 0.51±0.07; total cells 11.68±0.16 vs 8.79±0.75 in 0.5-month-old *Ddo*-KO and *Ddo*-WT, respectively). Data are presented as mean±SEM. ANOVA followed by Tukey *post hoc* test was used for statistical analysis. Four animals were used for each experimental group. Scale bar 100 and 50 μm for panoramic and inset image, respectively.

Figure 5. Active caspase 3 and 7 expression in the hippocampus and PFC of *Ddo*-KO mice. (A-L_1) Representative images showing activated caspase 3 (green) immunoreactivity in the CA1 field of hippocampus (A-F_1) and in *laminae* II/III of infralimbic cortex of *Ddo*-KO and *Ddo*-WT mice (G-L_1). Note the highest density of caspase 3 immunopositive puncta in stratum pyramidale (s.p.) of 3 and 6 months *Ddo*-KO vs. age-matched *Ddo*-WT mice. (M-X_1) Representative images of showing activated caspase 7 (green) immunoreactivity in the CA1 field of hippocampus (M-R_1) and in *laminae* II/III of infralimbic cortex (S-X_1) of *Ddo*-KO and *Ddo*-WT mice. Note the highest density of caspase 7 immunopositive puncta in stratum pyramidale (s.p.) of 3 and 6 months KO vs. age-matched *Ddo*-WT mice. Nuclei are counterstained with DAPI (4',6-diamidino-2-phenylindole). Images are representative of n=30 sections per mouse; n=3 mice per age and genotype. Scale bar: 100 μm (A-X), 50 μm A_1-X_1.

Figure 6. Age-dependent modifications of LTP and NMDAR subunit levels in the hippocampus of *Ddo*-KO mice. A) Superimposed pooled data showing the normalized changes in the field excitatory post-synaptic potential (fEPSP) slope (±SEM) in *Ddo*-KO and *Ddo*-WT mice at different ages induced by theta burst stimulation (TBS) (arrow). At 0.5 months, synaptic potentiation, measured 60 min after TBS application, was 35±7% above baseline (n=5) in mutant vs. 34±11% (n=5) in control slices ($p>0.05$). The magnitude of LTP was 67±8% (n=6) in 3-month-old *Ddo*-KO vs. 42±6% (n=5) in age-matched *Ddo*-WT ($p<0.001$); 31±10% (n=5) in 6-month-old *Ddo*-KO vs. 49±10% (n=6) in age-matched *Ddo*-WT ($p>0.05$), and 10±7% (n=6) in 12-month-old *Ddo*-KO vs. 31±6% (n=5) in age-matched *Ddo*-WT ($p<0.01$). Superimposed representative traces were recorded before and 1 h following LTP induction. B-C) Western blot analysis was performed on the postsynaptic TIF fraction from the hippocampus of 0.5, 1-, 3-, 6- and 12-month-old *Ddo*-WT and *Ddo*-KO mice using antibodies raised against (B) the GluN1, GluN2A and GluN2B subunits of the NMDAR and (C) GluA1 and GluA2 subunits of the AMPAR. Optical density values of all tested proteins were normalized to tubulin. Protein levels of *Ddo*-KO mice are expressed as % of age-matched *Ddo*-WT mice. Representative blots are shown for each protein detected. All values are expressed as mean±SEM. Data were analyzed by Student's t-test by comparing *Ddo*-KO with age-matched *Ddo*-WT mice. Genotypes are as indicated (n=6 per genotype; *$p<0.05$, **$p<0.01$, KO vs. the corresponding age-matched *Ddo*-WT mice group).

Figure 7. Normal AMPAR and NMDAR current-voltage relationship and age-dependent changes of the AMPAR/NMDAR ratio in *Ddo*-KO mice. A-D) Averaged normalized amplitudes of evoked

AMPAR-mediated or NMDAR-mediated current recorded from CA1 pyramidal neurons showing (A, B, C) no differences in current–voltage relationship between *Ddo*-KO and age-matched *Ddo*-WT neurons ($p>0.05$),and (D) in *Ddo*-KO mice at the three age stages ($p>0.05$). (A) At 0.5 months of age, the AMPAR/NMDAR-EPSC ratio was 0.56±0.11 (n=6) in *Ddo*-KO and 1.36±0.14 (n=7) in age-matched *Ddo*-WT mice ($p<0.001$); (B) 0.75±0.15 (n=6) in 3-month-old *Ddo*-KO and 1.55±0.18 (n=6) in age-matched *Ddo*-WT mice ($p<0.001$); (C) 1.36±0.19 (n=5) in 6-month-old *Ddo*-KO and 1.18±0.13 (n=6) in age-matched *Ddo*-WT mice ($p>0.05$). The representative traces of either AMPAR-EPSCs or NMDAR-EPSCs are shown on top of each histogram. ***$p<0.001$.

Figure 8. Increased sensitivity to phencyclidine-induced prepulse inhibition deficits in *Ddo*-KO mice. Prepulse inhibition of the acoustic startle response was measured in 6-month-old mice treated with (A) 6 mg/kg PCP (n=8 *Ddo*-WT; n=8 *Ddo*-KO) or saline (n=10 *Ddo*-WT; n=10 *Ddo*-KO), (B) 12 mg/kg PCP (n=10 *Ddo*-WT; n=10 *Ddo*-KO) or saline (n=10 WT; n=10 KO). Percentage of PPI was used as dependent variable. *$p<0.05$, **$p<0.01$ compared to vehicle-treated groups (two-way ANOVA with repeated measures). All values are expressed as mean±SEM.

Figure 1 revised

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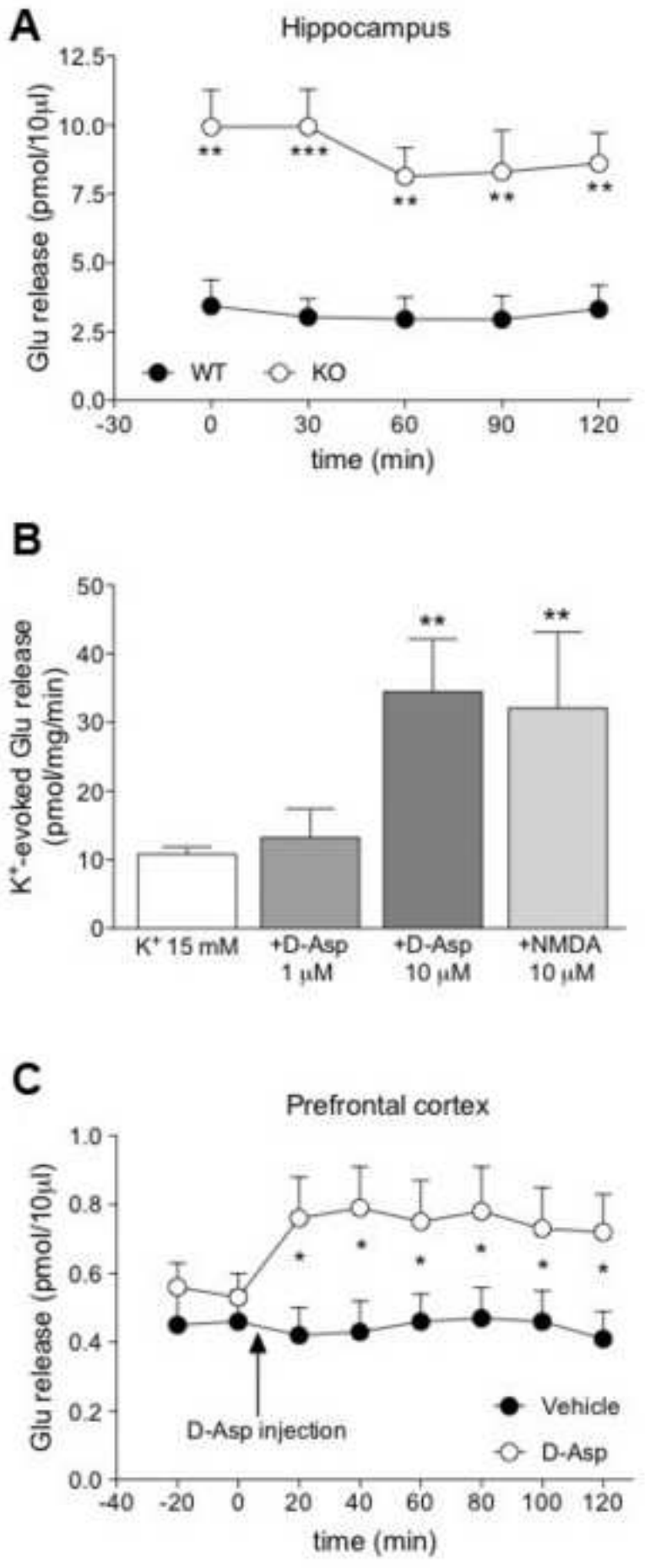

Figure 2

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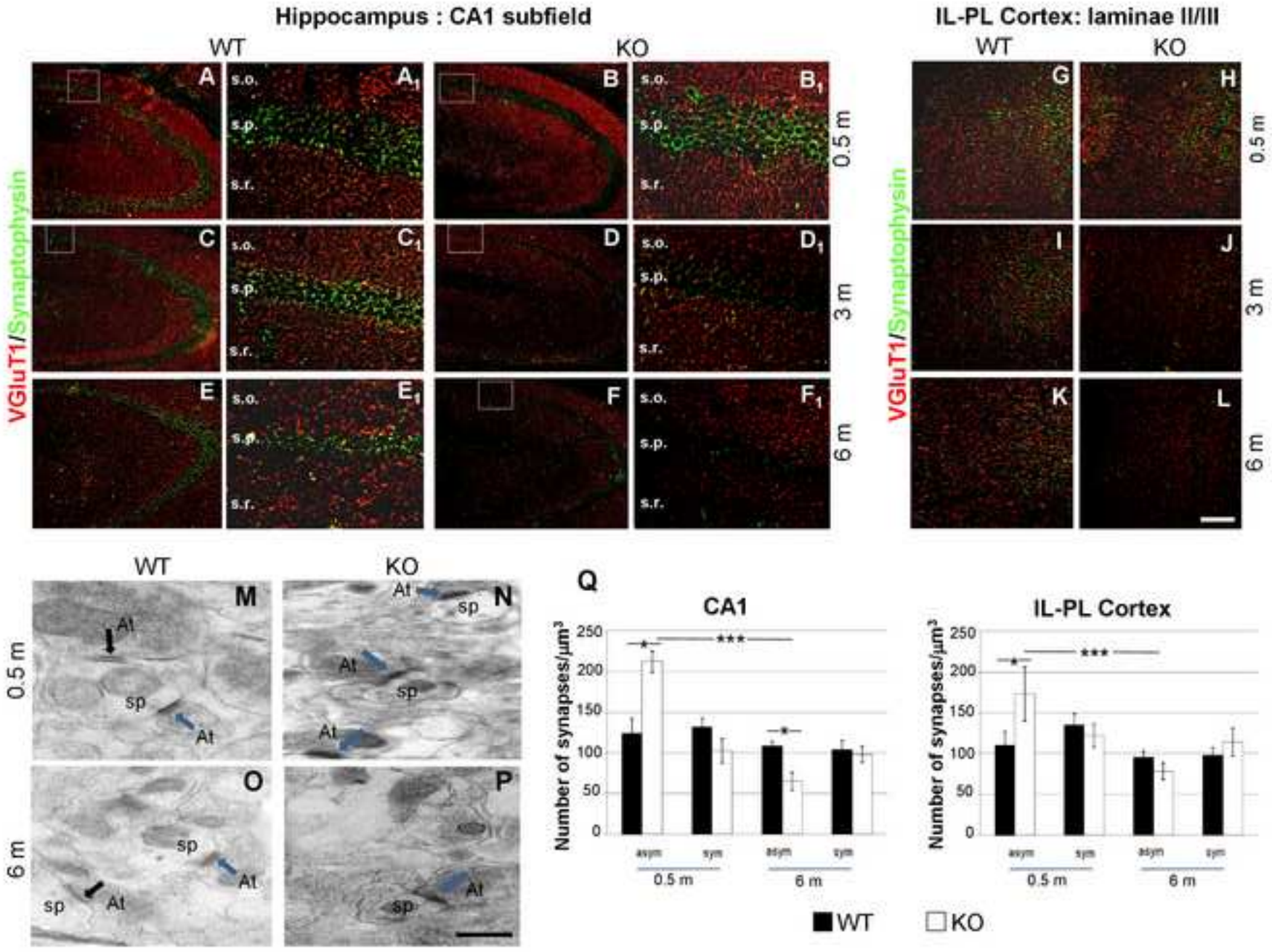

Figure 3

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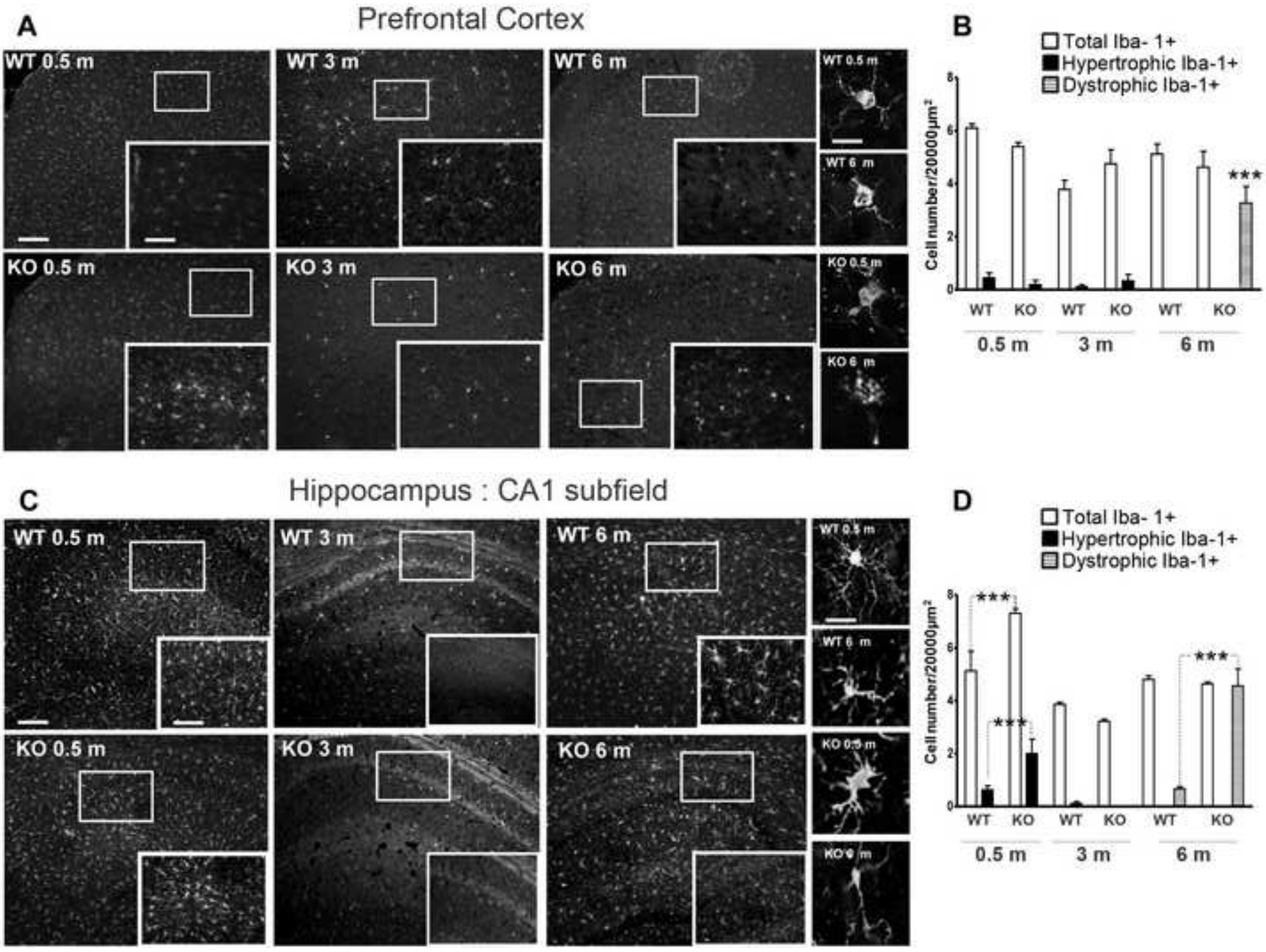

Figure 4 new

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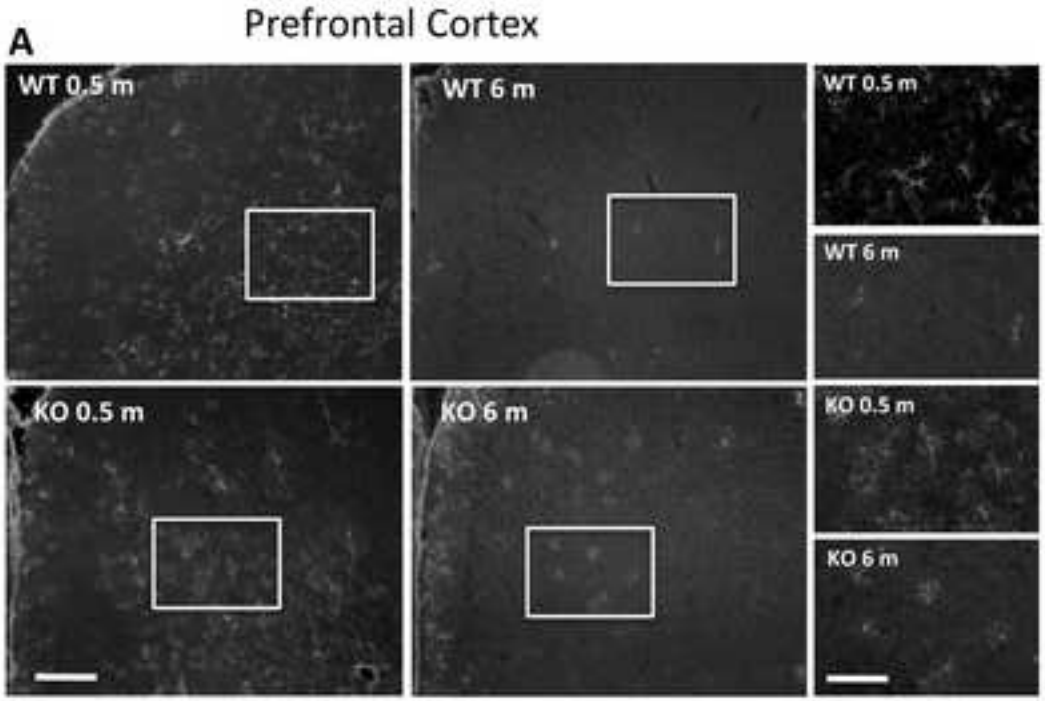

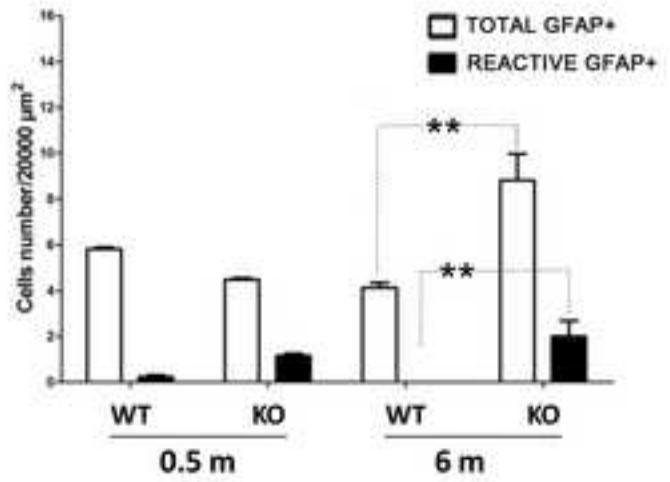

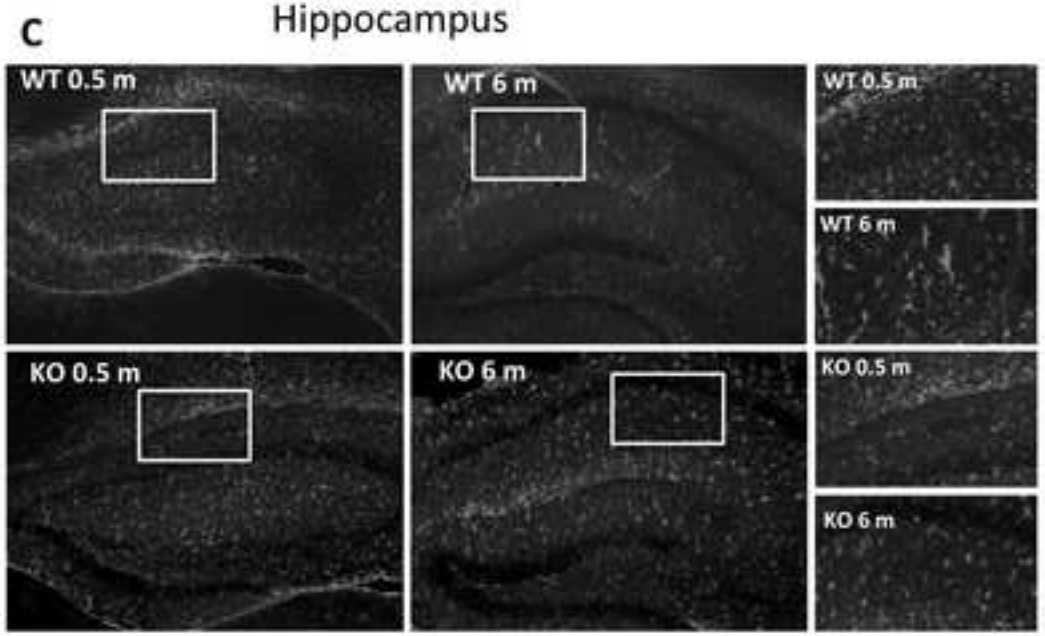

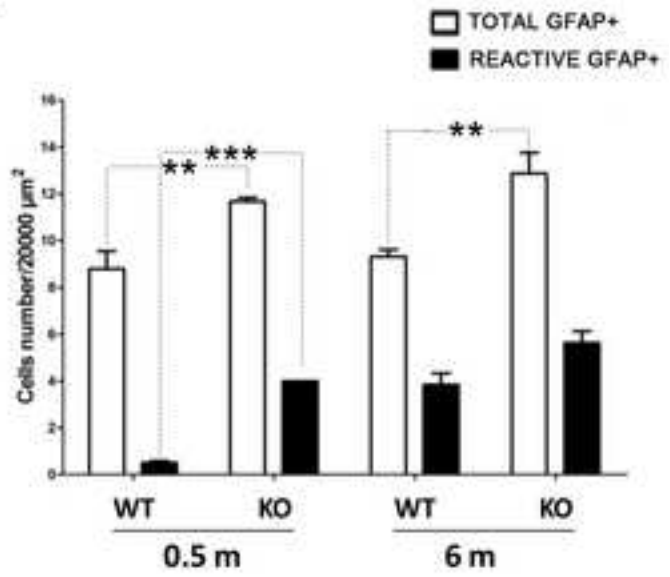

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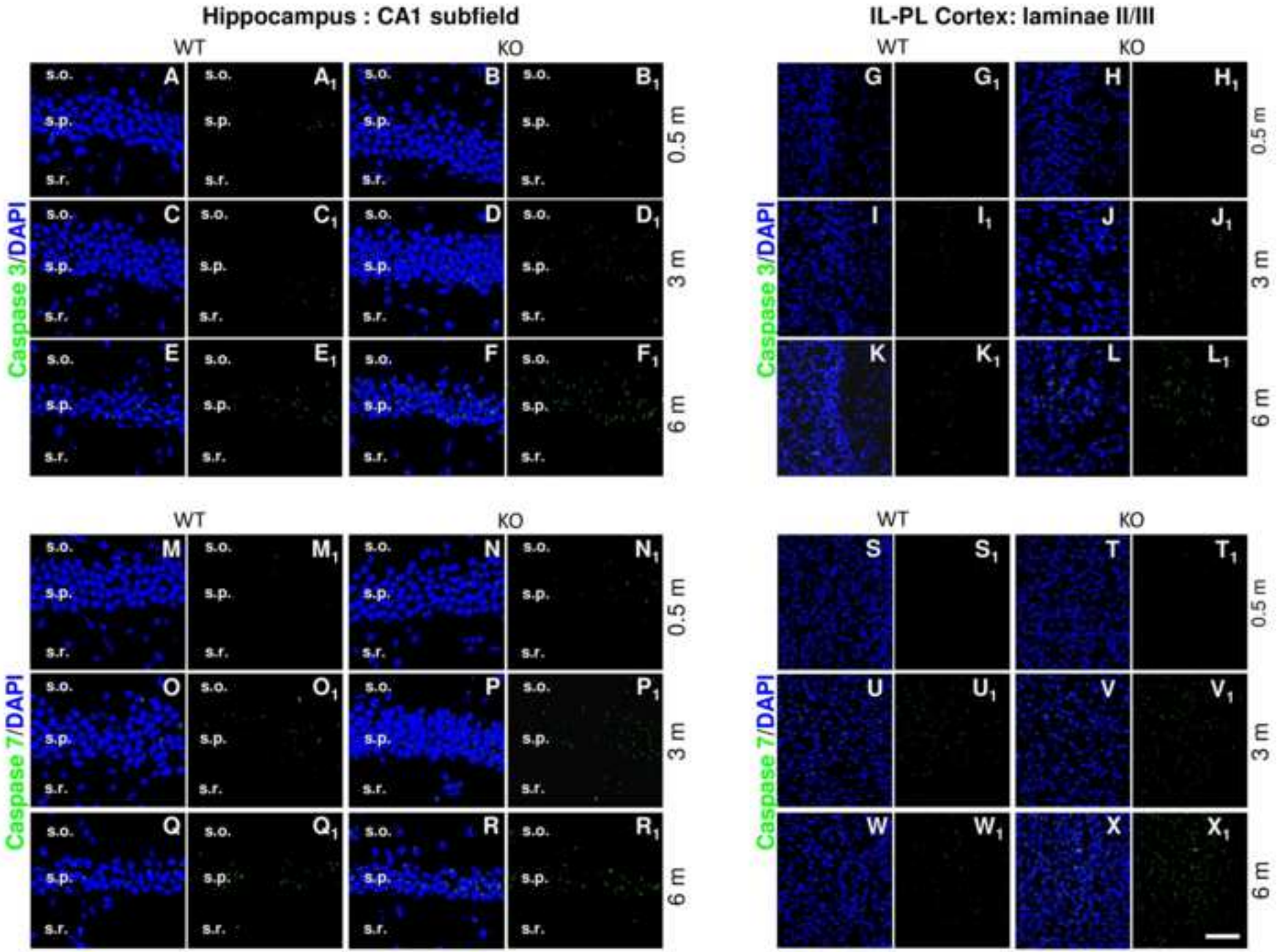

Figure 6

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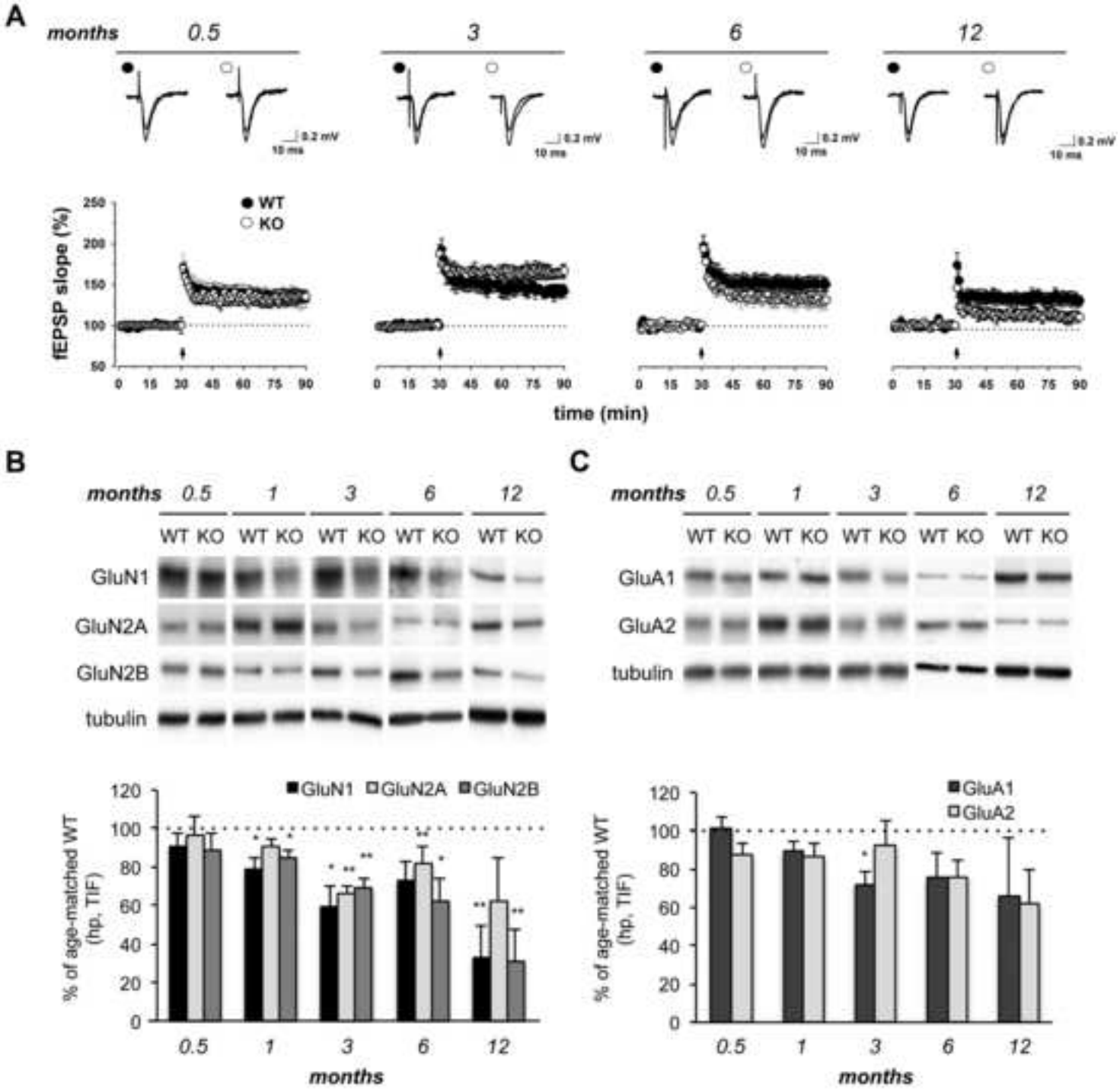

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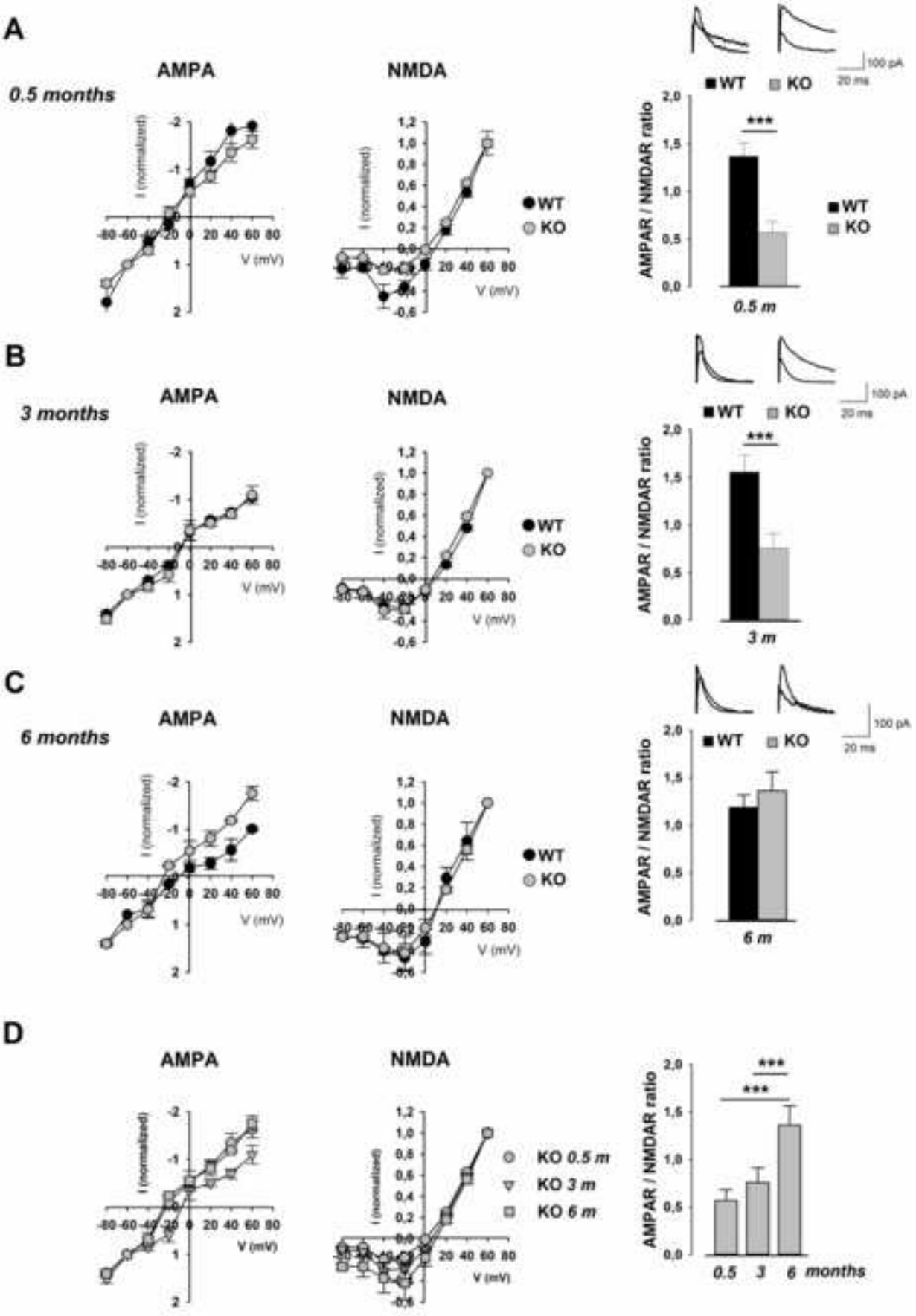

Figure 8 revised

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