Estradiol reverses excitatory synapse loss in a cellular model of neuropsychiatric disorders

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Running title: Estrogens rescue DISC1-synaptic deficits.

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Abstract

Loss of glutamatergic synapses is thought to be a key cellular pathology associated with neuropsychiatric disorders including schizophrenia (SCZ) and major depressive disorder (MDD). Genetic and cellular studies SCZ and MDD using *in vivo* and *in vitro* systems have further supported a key role for dysfunction of excitatory synapses in the pathophysiology of these disorders. Recent clinical studies have demonstrated that the estrogen, 17β-estradiol can ameliorate many of the symptoms experienced by patients. Yet, to date, our understanding of how these beneficial effects are exerted by 17β-estradiol is limited. In this study, we have tested the hypothesis that 17β-estradiol can restore excitatory synapses number in a cellular model that recapitulates the loss of synapses associated with SCZ and MDD. Specifically, we show that in cortical neurons with reduced dendritic spine density, produced by either ectopically expressing wildtype or mutant Disrupted in Schizophrenia (DISC1) constructs, that acute treatment with 17β-estradiol increased spine density to control levels. Furthermore, 17β-estradiol causes a redistribution of both exogenous and endogenous DISC1, a mechanism whereby DISC1's inhibitory effects on synapse formation is thought to occur. In addition, we also observe an increased targeting of PSD-95 and kalriin-7, which form a signalsome with DISC1, following 17β-estradiol treatment. Taken together, out data indicates that estrogens can restore lost excitatory synapses caused by altered DISC1 expression, potentially through the bi-directional trafficking of DISC1 and its interacting partners. These data highlight the possibility that estrogens exert their beneficial effects in SCZ and MDD, in part by modulating excitatory synaptic number.

Introduction

Glutamatergic synapse dysfunction is thought to be a key cellular hallmark of a number of neuropsychiatric disorders including schizophrenia (SCZ) and major depressive disorder (MDD)[1-4]. In the mammalian forebrain, the majority of excitatory glutamatergic synapses occur on specialized structures known as dendritic spines, which protrude off and decorate the dendrite of pyramidal neurons[4, 5]. Dendritic spines house the post-synaptic density (PSD), where a number of key synaptic proteins involved in synaptic transmission and signaling are located[4, 5]. *Post-mortem* studies have also shown that there are reduced dendritic spine numbers in the brains of patients with SCZ and MDD[2, 6-8]. Consistent with this genetic studies have linked a number of genes encoding for synaptic proteins or for proteins that regulate synapse structure or function with neuropsychiatric disorders including SCZ and MDD[1, 2, 4, 9-11]. Therefore, it has been suggested that regulating synapse protein and dendritic spine number may be a viable therapeutic strategy in the treatment of SCZ and/or MDD[6, 11, 12].

The neurosteroid, 17β-estradiol, has been shown to be a potent neuromodulator, having positive effects cognitive processes including learning and memory as well as mood[13, 14]. The effect of 17β-estradiol, the principal biologically active estrogen, on cognition is thought to be driven, in part, by activation of specific signaling pathways resulting in alternations in dendritic spine number and the trafficking of key synaptic proteins (reviewed in 13). Recently, clinical studies have shown that treatment with 17β-estradiol has beneficial effects for patients diagnosed with SCZ or MDD, when given as an adjunct treatment to ongoing antipsychotic or antidepressant therapies[15-19]. However, the molecular and cellular mechanisms by

which 17β-estradiol exert these beneficial effects are currently unknown. One possibility is that 17β-estradiol exerts its beneficial effects via the modulation of glutamatergic synapses[13, 20, 21]. However, this has not been tested directly in a cellular model of disease.

In this study, we have utilized a cellular model of neuropsychiatric disorders, in the form of manipulating the expression levels of the disrupted-in-schizophrenia 1 (DISC1) protein. The *DISC1* gene has been implicated in the pathophysiology of SCZ and MDD[22-26]. While the contribution of this gene to the etiology of SCZ and MDD remains unclear and somewhat controversial[27, 28], results from animal and cellular models have demonstrated that altering the expression levels of DISC1 protein results in a loss of dendritic spine density[29-32], a result consistent with that seen in postmortem studies of patients with SCZ or MDD[2, 4, 11]. Therefore, in this study, we manipulated the expression of DISC1 to reduce dendritic spine density[29, 33]. Subsequently, we treated cells with 17β-estradiol to explore whether this neurosteroid could modulate glutamatergic synapses in a cellular model, recapitulating aspects of the pathophysiology associated with SCZ and MDD. A number of studies have suggested that the aggregation of DISC1 might be important for psychiatric disease. An original study identified high molecular weight insoluble aggregates of DISC1 in patients specifically diagnosed with major mental illness including SZZ and MDD[34, 35]. Thus, we also investigated whether 17β-estradiol altered mutant or wildtype DISC1 aggregates, and further examined the sub-cellular distribution of endogenous DISC1 and its synaptic interacting proteins following treatment. Our results demonstrate that acute treatment with 17β-estradiol is sufficient to restore dendritic spine deficits to basal levels, whilst concurrently reducing DISC1 aggregates. Moreover, we go on to show that 17β-estradiol can

modulate DISC1 enrichment at synapses, as well as its interacting partner, kalirin-7, a Rac-GEF known to regulate spine density[29]. Thus, these data suggest that the mechanism by which 17β-estradiol is beneficial in the treatment of SCZ and MDD, may in part be driven by its ability to regulate excitatory glutamatergic synapses.

Methods

Reagents

Antibodies used: GFP chicken polyclonal (ab13972; Abcam; 1:10,000); HA mouse monoclonal (ab18181; Abcam; 1:200); kalirin rabbit polyclonal (07-122; Millipore; 1:500); PSD-95 mouse monoclonal antibody (clone K28/43; 73-028; NeuroMab; 1:1000); DISC1 440 rabbit polyclonal (used at 1:250) has been previously described[36, 37]. The antigenic peptide was RTPHPEEEKSPLQVLQEWD, which is identical in human, mouse and rat. DISC1–440 consistently recognizes four major isoforms from rat brain lysates, including two bands at 130 kD and two around 100 kD[36, 37]. 17β-estradiol (E8875) was from Sigma. HA-DISC1WT and HA-DISC1ΔCT constructs were kind gifts from A. Sawa (Johns Hopkins University); generation and validation of these constructs have previously been described[29, 38].

Neuronal culture and transfections

Cortical neuronal cultures, consisting of mixed sexes, were prepared from E18 Sprague-Dawley rat embryos as described previously[39]. Animals were habituated for 3 days before experimental procedures, which were carried out in accordance with the Home Office Animals (Scientific procedures) Act, United Kingdom, 1986. Cells were plated onto 18 mm glass coverslips (No 1.5; 0117580, Marienfeld-Superior GmbH & Co.), coated with poly-D-lysine (0.2mg/ml, Sigma), at a

density of $3x10^5$/well equal to 857/mm^2. Neurons were cultured in feeding media: neurobasal medium (21103049) supplemented with 2% B27 (17504044), 0.5 mM glutamine (25030024) and 1% penicillin:streptomycin (15070063) (all reagents from Life technologies). Neuron cultures were maintained in presence of 200 µM D,L-amino-phosphonovalerate (D,L-APV, ab120004, Abcam) beginning on DIV (days *in vitro*) 4 in order to maintain neuronal health for long-term culturing and to reduce cell death due to excessive Ca^{2+} cytotoxicity via over-active NMDA receptors[39]. We have previously shown that the presence or absence of APV in the culture media does not affect E2's ability to increase spine linear density[40]. Half media changes were performed twice weekly until desired age (DIV 23-25). The primary cortical neurons were transfected with eGFP at DIV 23 for 2 days, using Lipofectamine 2000 (11668027, Life Technologies)[39]. Briefly, 4-6 µg of plasmid DNA was mixed with Lipofectamine 2000 and incubated for 4-12 hours, before being replaced with fresh feeding media. Transfections were allowed to proceed for 2 days after which cells were used for pharmacological treatment or immunocytochemistry (ICC).

Pharmacological treatments of neuron culture

All pharmacological treatments were performed in artificial cerebral spinal fluid (aCSF): (in mM) 125 NaCl, 2.5 KCL, 26.2 $NaHCO_3$, 1 NaH_2PO_4, 11 glucose, 5 HEPES, 2.5 $CaCl_2$, 1.25 $MgCl_2$, and 0.2 APV). 17β-estradiol was dissolved in DMSO at a concentration of 10 mM, serially diluted to a 10X working concentration in aCSF, and applied directly to neuronal cultures. Final concentration of solvent was < 0.01%; vehicle control was made up of solvent lacking compound, diluted as test compounds. Treatments were allowed to proceed for 30 minutes before being lysed for Western blotting or fixed for ICC.

Immunocytochemistry (ICC)

Neurons were washed in PBS and then fixed in either 4% formaldehyde/4% sucrose PBS for 10 minutes at room temperature followed by incubation in methanol pre-chilled to -20°C for 10 minutes at 4°C, or only in methanol (-20°C) for 20 minutes at 4°C. Fixed neurons were then permeabilized and blocked simultaneously (2% Normal Goat Serum, 5425S, New England Biolabs and 0.1% Triton X-100) before incubation in primary antibodies overnight and subsequent incubation with secondary antibodies the following day[39]. In the green/magenta colour scheme, co-localization is indicated by white overlap.

Quantitative Analysis of Spine Morphologies and Immunofluorescence

Confocal images of double-stained neurons were acquired with a Leica SP-5 confocal microscope using a 63x oil-immersion objective (Leica, N.A. 1.4) as a z-series, or with a Zeiss Axio Imager Z1, equipped with an ApoTome using a 63x oil-immersion objective (Carl Zeiss, N.A. 1.4). Two-dimensional maximum projection reconstructions of images were generated and linear density calculated using ImageJ/Fiji (https://imagej.nih.gov/ij/)[39]. Morphometric analysis was performed on spines from two dendrites (secondary or tertiary branches), totalling 100 µm, from each neuron. Linear density and total gray value of each synaptic protein cluster was measured automatically using MetaMorph Software (Molecular Devices)[39]. Cultures directly compared were stained simultaneously and imaged with the same acquisition parameters. For each condition, 10-16 neurons from at least 3 separate experiments were used. Experiments were carried out blind to condition and on sister cultures.

Biochemistry cell fractionation

Crude synaptosome fractions were prepared from DIV 25 neurons following treatment with 17β-estradiol or vehicle for 30 minutes. Cells were lysed in homogenisation buffer (320 mM sucrose; 5 mM Na4P2O7; 1 mM EDTA pH 8; and 10 mM HEPES pH 7.4 + protease inhibitors) and subsequently passed through a 21 gauge needle 15 times. Cell lysate was then centrifuged to remove the nuclear fraction and large cell organelles (P1 fraction), and yield the extranuclear fraction (S1) A portion of the S1 fraction was kept and the remaining was subjected to further fractionation by an additional spin. This yielded the cytosolic (S2) and crude synaptosome (P2) fractions; the P2 fraction was resuspended in homogenisation buffer. Sample buffer was added to all samples, which were then denatured for 5 minutes at 95°C and stored at -80°C until used further. All samples were subsequently separated by SDS-PAGE and analyzed by Western Blotting with kalirin, DISC1, PSD-95 and β-actin antibodies. Quantification of bands was performed by measuring the integrated intensity of each band and normalizing to β-actin, for protein loading, using Image J.

Statistical Analysis

All statistical analysis was performed in GraphPad. Differences in quantitative immunofluorescence, dendritic spine number were identified by Student's unpaired t-tests, or for comparisons between multiple conditions the main effects and simple effects were probed by one-way-ANOVAs with Tukey correction for multiple comparisons. Error bars represent standard errors of the mean.

Results

Exogenous expression of C-terminal DISC1 mutant causes loss of dendritic spines and aggregates within dendrites.

Disruption in glutamatergic transmission and abnormal dendritic spine morphology and number is a cellular phenotype shared across a number of neurodevelopmental and psychiatric disorders[4, 22]. DISC1, a risk factor associated with mental illness, is a scaffold protein that is enriched at synapses; it has been shown to regulate dendrite spine morphology, number and glutamatergic transmission[22, 24]. DISC1 was originally identified through analysis of a large Scottish family[23] where a balanced chromosomal translocation, associated with psychiatric illness, is thought to lead either to a loss of DISC1 or the formation of a dominant-negative C-terminally truncated DISC1 protein[22, 25]. The C-terminal truncation has been used extensively to model DISC1 pathology in cellular and transgenic models. However, the effect of the C-terminal DISC1 mutation on dendritic spine density has not been investigated. Therefore, we overexpressed either HA-tagged full-length DISC1 (HA-DISC1) or a C-terminal truncated DISC1 mutant (HA-DISC1ΔCT) in DIV 24-26 primary neurons. As expected, neurons expressing HA-DISC1 had a reduced linear spine density compared to control cells (**Figure 1A & B**). Remarkably, neurons exogenously expressing HA-DISC1ΔCT also displayed reduced spine density compared to control (dendritic spine linear density/10 µm): control, 6.3±0.6; HA-DISC1, 2.87±0.42; HA-DISC1ΔCT, 2.54±0.34; ** = $p < 0.01$; **Figure 1A & B**). Recent studies have also indicated that the aggregation of DISC1 may be central to its dominant negative effects and thus, contribute its pathophysiological role[34, 41, 42]. In cortical neurons, endogenous DISC1 is localized in punctate structures along

dendrites and within dendritic spines, indicating an enrichment at synapses **(Figure 1C (i))**. Consistent with previous reports HA-DISC1 formed large aggregates within dendrites. Interestingly, HA-DISC1ΔCT was also observed to accumulate long dendrites similar to HA-DISC1 **(Figure 1C (ii) & (iii))**. Taken together, these data reveal that like wildtype DISC1, DISC1ΔCT causes a reduction in dendritic spine density, and accumulate in dendrites where they appear to forms aggregates.

Modulation of Dendritic spine density by 17β-estradiol in neurons expressing wildtype or C-terminal truncated DISC1.

Previous studies have demonstrated that 17β-estradiol can increase dendritic spine density in cortical neurons[40, 43], but whether this occurs within neurons recapitulating a disease relevant reduction in spine number is unknown. Therefore, we next asked whether acute treatment with 17β-estradiol could also restore dendritic spine density in neurons overexpressing HA-DISC1 or HA-DISC1ΔCT. Under control conditions, 17β-estradiol increased spine density compared to vehicle treatment **(Figure 2A, left panel)**. In neurons expressing HA-DISC1, treatment with 17β-estradiol resulted in an increase in the number of spines **(Figure 2A, middle panel)**. Similarly, in neurons ectopically expressing HA-DISC1ΔCT, spine density was again increased following 17β-estradiol treatment **(Figure 2A, right panel)**. Interestingly, following treatment, spine linear density was similar to untreated control condition in both HA-DIC1 and HA-DISC1ΔCT expressing cells (dendritic spine linear density/10 μm): control, 5.8±0.21; control + 17β-estradiol, 7.27±0.44; HA-DISC1, 3.9±0.2; HA-DISC1 + 17β-estradiol, 5.22±0.34; HA-DISC1ΔCT, 3.4±0.36; HA-DISC1ΔCT + 17β-estradiol, 5.5±0.37; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; **Figure 2 & B**). Collectively, these data demonstrate that 17β-estradiol is capable of

modulating dendritic spine linear density to a level similar to control in neurons expressing either wildtype or mutant DISC1.

Acute treatment with 17β-estradiol reduces aggregation of wildtype and mutant DISC1.

Recent work has suggested that DISC1 forms aggregates under physiological and pathological conditions[34, 41, 42]. The formation of aggregates has been proposed to contributes to DISC1's cellular function and the cellular pathology associated with the altered expression of this protein[34, 41, 42]. Intriguingly, reducing DISC1 aggregation has been linked with a reduction in cellular pathology[41, 44]. However, whether DISC1 aggregation is linked with the loss of dendritic spine density induced by overexpression, and whether modulating DISC1 aggregation could influence synaptic deficits is not known. As 17β-estradiol could restore aberrant spine density back to a level similar to basal conditions, we tested the hypothesis that it may also reduce the extent to which ectopic DISC1 aggregated. Under control conditions, both HA-DISC1 and HA-DISC1ΔCT could be observed as large clusters within the dendrite (**Figure 3A**). However, after treatment with 17β-estradiol, both HA-DISC1 and HA-DISC1ΔCT clustering was reduced, with smaller and fewer clusters evident within dendrites (**Figure 3A & B**). Taken together, these data indicate that concurrent with the ability to increase dendritic spine density, 17β-estradiol reduces clustering of exogenous wildtype and mutant DISC1.

Acute 17β-estradiol treatment restores spine loss induced by reduced Disc1 expression.

To ensure that the effects observed above were no due to an artifact of overexpressing Disc1 constructs, we employed a second approach to alter Disc1 function. Previous studies have demonstrated that long term shRNA-mediated knockdown of Disc1 results in a loss of dendritic spine linear density in both *in vitro* and *in vivo* systems[29, 33]. Thus, reducing Disc1 expression levels has been suggested to be an approach to induce a cellular phenotype relevant for neurodevelopmental and psychiatric disorders[33]. To this end, we utilized a previously validated shRNA construct for rat Disc1[29, 33, 38]. The Disc1_shRNA construct was able to knockdown exogenous HA-tagged rodent Disc1 in hEK293 cells, confirming it specificity for the protein **(Figure 4A + B)**. We next expressed Disc1_shRNA or control construct in primary cortical neurons for 7 days; this resulted in a significant reduction of spine linear density by ~35% compared to untreated control **(Figure 4C + D)**, consistent with previous studies[33]. Treatment of control neurons with 10 nM 17β-estradiol increased spine density by ~40% within 30 minutes **(Figure 4C + D)**. Interestingly, treatment of expressing Disc1_shRNA neurons with 17β-estradiol was sufficient to restore linear spine density to a level similar to control levels (dendritic spine linear density (per 10 µm): control, 5.1±0.3; control + 17β-estradiol, 7.25±0.49; Disc1_shRNA, 3.4±0.35; Disc1_shRNA + 17β-estradiol, 4.95±0.27; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; **Figure 4C + D**). These data demonstrate that 17β-estradiol is capable of modulating spine linear density in neurons with reduced Disc1 levels.

Redistribution of DISC1 and kalirin-7 following acute 17β-estradiol treatment.

As our data indicated that 17β-estradiol could modulate the sub-cellular distribution of exogenous wildtype and mutant DISC1, we reasoned that it may also influence the distribution of endogenous DISC1 and it binding partners. Therefore,

we evaluated whether acute exposure to 17β-estradiol could alter the sub-cellular localization of the DISC1/PSD-95/kalirin-7 signalosome. In support of this idea we have recently shown that 17β-estradiol causes the rapid redistribution of several synaptic proteins, including PSD-95, from dendrites into spines[43]. Importantly, the ability of DISC1 to regulate spinogenesis is thought to be mediated by its interactions with key synaptic proteins, including PSD-95 and kalirin-7 a Rac-GEF[29]. We first tested this by examining whether levels of DISC1/PSD-95/kalirin-7 were altered following treatment with 17β-estradiol. Western blotting of whole cell lysates of neurons treated with 17β-estradiol for 0 or 30 minutes, revealed no change in overall expression of DISC1, kalirin-7 or PSD-95 (**Figure 5A**; 'whole cell lysate'). Next, we examined the enrichment of these proteins in crude synaptosomal (P2) and cytosol (S2) fractions. This revealed that 17β-estradiol caused a loss of DISC1 specifically within the P2 fraction, with a concurrent increase in S2 fraction (**Figure 5A & B**). Conversely, following treatment with 17β-estradiol, the enrichment of both kalirin-7 and PSD-95 in crude synaptosomal fractions (P2) was increased, but decreased in S2 fractions. (**Figure 5A & B**). To demonstrate that DISC1 was being removed from post-synaptic compartments, we further examined the content of this protein within dendritic spines with or without treatment. As suggested by our biochemical data, assessment of endogenous DISC1 content within spines was significantly reduced following treatment with 17β-estradiol (**Figure 5C**). This was further highlighted by line scans analysis across spines, as following 17β-estradiol treatment, the intensity of DISC1 staining within dendritic spines was reduced (**Figure 5D**). Thus, these data indicate that 17β-estradiol specifically removes DISC1 from dendritic spines into non-synaptic regions.

We next sought to determine whether kalirin-7 and PSD-95 were being trafficked to the same location following 17β-estradiol treatment, by assessing the number of kalirin-7 and co-localised PSD-95/kalirin-7 puncta. Consistent with our previous data, we observed an increase in the density of PSD-95 puncta **(Figure 6A)**, which we have previously shown to represent an increase of synaptic sites[43]. Similarly, we found that challenge with 17β-estradiol resulted in an increase in total kalirin-7 puncta (**Figure 6A & B**). Under control conditions, ~65% of kalirin-7 puncta were positive for PSD-95; a similar level of co-localization was also seen following treatment with 17β-estradiol (**Figure 6A & B**). Consistent with these data, we found that kalirin-7 intensity within spines increases primarily following 17β-estradiol treatment (**Figure 6C**). The enrichment of kalirin-7 within spines following treatment could also be illustrated using line scan analysis across dendritic spines (**Figure 6D**), consequently supporting our biochemical data in demonstrating that a subpopulation of kalirin-7 was being trafficked to synaptic regions following 17β-estradiol treatment. Taken together, these data demonstrate that 17β-estradiol induces a bi-directional trafficking of DISC1, kalirin-7 and PSD-95, with the former protein having a reduced synaptic enrichment, and the latter two proteins increasing in their synaptic localization.

Discussion

In this study, we have tested the hypothesis that 17β-estradiol can restore excitatory synapses number in a cellular model that recapitulates the loss of synapses associated with SCZ and MDD. We show that in cortical neurons with reduced dendritic spine density produced by manipulating DISC1, acute treatment with 17β-estradiol increased spine density to control levels. Treatment with 17β-

estradiol was also able to reduce the extent to which ectopic wildtype and mutant DISC1 aggregated to. And finally we found that 17β-estradiol causes a redistribution of DISC1, as well as PSD-95 and kalrin-7, which form a signalsome with DISC1. Taken together, out data indicates that estrogens can restore lost excitatory synapses caused by altered DISC1 expression, potentially through the bi-directional trafficking of DISC1 and its interacting partners.

Recent clinical studies have demonstrated that adjunct treatment with 17β-estradiol and estrogen-based compounds ameliorate positive, negative and cognitive symptoms experienced by patients diagnosed with schizophrenia or depression[15-19]. Hitherto, the exact molecular and cellular mechanisms by which estrogens exert these beneficial effects are not understood. The beneficial effects of 17β-estradiol in disorders such as SCZ and MDD have been proposed to occur through the modulation of monoamine transmitter systems such as dopamine and 5-HT, or via an anti- inflammatory mechanism[16, 19, 21, 45]. However, it has also been suggested that the beneficial effects of 17β-estradiol may also be in part conferred through the modulation of glutamatergic signaling[13, 20, 21]. This is supported by animal studies which show that 17β-estradiol enhances performance on a number of cognitive tasks, including attention and learning and memory tasks in healthy animals[13, 14, 46] as well as models of psychosis[16, 47-49]. Importantly, it is the ability of 17β-estradiol to modulate both glutamatergic and GABAergic systems that underlies these enhancing effects[40, 43, 50-53]. This supports the rationale for examining whether 17β-estradiol could regulate excitatory synapses in a disease-relevant cellular model.

It is important to consider the use of DISC1 as the mediator of cellular pathology in this study. DISC1 had emerged as a candidate risk factor for major mental illnesses, including SCZ, autism spectrum disorder, bipolar disorder and

MDD[24]. While DISC1 may not directly contribute to the etiology of these disorders[24, 27, 28], it has been argued that DISC1-based cellular systems are useful in understanding cellular mechanisms relevant for neuropsychiatric disorders[22], and in exploring potential therapeutic agents[33]. Taking advantage of the ability of DISC1 to induce spine loss, data presented in this study provides evidence that 17β-estradiol is capable of increase excitatory synapse number in a disease-relevant cellular model. Collectively, these data indicates that *in vivo*, the beneficial effects of estrogens could be driven in part by the regulation of glutamatergic synapses. It should be noted, however, that these results do not negate the possibility that 17β-estradiol also modulates multiple other systems, and this also contributes to its beneficial effects in SCZ and MDD.

A novel finding of our study is that ectopic expression of a C-terminal truncated form of DISC1, thought to represent the consequence of the original identified translocation[22, 38], also induces the loss of dendritic spines. Both DISC1 and DISC1ΔCT constructs formed large clusters, or aggregates, along dendrites, similar to previous reports[34, 41]. The aberrant formation of DISC1 aggregates is a key event that results in disruption of a number of cellular processes. Interestingly, our data indicates that concurrent with a return of spine density to basal levels, 17β-estradiol, also reduces the extent to which DISC1 forms aggregates. While a direct link between DISC1 clustering and spine deficits has not been established, it will be interesting in future studies to ascertain whether these are two separate events, or whether 17β-estradiol's ability to restore spine density to basal levels is also connected to its ability to reduced DISC1 clustering.

In support of such an idea, we show that 17β-estradiol is capable of altering the distribution of endogenous DISC1 as well. Interestingly, our data indicates 17β-

estradiol reduced the synaptic content of endogenous DISC1. This is consistent with previous data indicating that endogenous DISC1 acts to limit spine formation[29]. Activity-dependent signaling reduces the extent to which DISC1 interacts with PSD-95/kalirin-7 which in turns allows for an increase in spine size and number[29, 33]. Consistent with this model, we show that 17β-estradiol caused the bi-directional redistribution of the DISC1/PSD-95/kalirin-7 signalsome. Whereas the presence of endogenous DISC1 at synapses is reduced following 17β-estradiol treatment, both PSD-95 and kalirin-7 are increased at synapses. This is consistent with our previous data showing that PSD-95 is targeted to nascent spines formed by 17β-estradiol[43]. An increased presence of kalirin-7 at synapses would also be consistent with an increase in spine formation. Indeed, we have previously shown that the synaptic localization of kaliirn-7 is important for both spine maintenance and formation[54]. As PSD-95 is required for correct kalrin-7 regulation of dendritic spines, the observed increased co-localized PSD-95/kalirin-7 puncta would indicate that this complex may contribute to the formation of nascent spines. Therefore, in this model, 17β-estradiol acts to release the complex between DISC1 and PSD-95/kalirin-7, in order to allow it to engage with the machinery required for spine formation.

The data presented in this study support a role for estrogenic-modulation of dendritic spines as a potential cellular mechanism by which its beneficial effects in SCZ and MDD may occur. Owing to the link between estrogenic regulation of spines with improvement in cognitive function, especially in learning and memory[40, 52, 53], as well as emerging data that estrogen-based compounds improve attention and working memory in male and female SCZ patients[15, 17, 19, 21], it is intriguing to suggest that this is part of the mechanisms that underlies the beneficial actions of estrogens. It is likely that the regulation of spines by 17β-estradiol is only part of the mechanism

underlying its beneficial actions. Critically, future studies using genetic models of disease or patient induced pluripotent stem cell models should allow for a more faithful recapitulation of both the cellular and complex genetic architecture associated with these disorders. Nevertheless, the data in this study support further investigations into estrogenic modulation of glutamatergic signaling and highlights this mechanism as having relevance for the therapeutic potential of estrogens in SCZ and MDD.

In conclusion, our data indicates that estrogens can restore lost excitatory synapses caused by altered DISC1 expression, potentially through the bi-directional trafficking of DISC1 and its interacting partners. These data highlight the possibility that estrogens exert their beneficial effects in SCZ and MDD, in part by modulating excitatory synaptic number.

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Author Contributions

F.E., A.B.P., J.M., N.J.B., P.P., K.J.S. and D.P.S. designed experiments. F.E., A.B.P., P.R., K.J.S. and D.P.S. performed all experiments and subsequent analysis.

J.M., and P.P. produced or oversaw production of reagents, J.M., N.J.B., P.P. and D.P.S. wrote the manuscript.

Conflict of interests

J.M. and N.J.B. are or were full-time employees and shareholders of AstraZeneca**.**

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Figure Legends:

Figure 1. C-terminal truncated DISC1 induces spine loss and aggregates within dendrites. (A) Representative images of cortical neurons (DIV 26) transfected with GFP alone, GFP + HA-DISC1 or GFP + HA-DISC1ΔCT. **(B)** Quantification of spine linear density. Overexpression of HA-DISC1 causes a reduction of spine density as previously reported; exogenous expression of DISC1ΔCT also causes a reduction in

spine linear density ($F(2,46)=21.54$, $p < 0.001$, Tukey Post Hoc, ***, $p < 0.001$, one-way ANOVA; n = 15-17 cells per condition from 4 independent cultures). **(C)** Endogenous DISC1 could be observed in discreet punctate strictures within dendrites and juxtaposed to dendrites. In comparison, both wildtype and ΔCT forms of DISC1 were found to aggregate within dendrites. Scale bar = 5 µm.

Figure 2. Acute 17β-estradiol (E2) treatment increases dendritic spine linear density in neurons overexpressing HA-DISC1 or HA-DISC1ΔCT. (A) Representative images of cortical neurons (DIV 26) transfected with GFP alone (Ctl), GFP + HA-DISC1 or GFP + HA-DISC1ΔCT and treated with vehicle (Veh) or 17β-estradiol (E2) or not. **(B)** Quantification of spine linear density. treatment of Ctl cells with E2 resulted in an increase in spine number. Overexpression of HA-DISC1 HA-DISC1ΔCT caused a significant reduction in spine linear density. Treatment with 17β-estradiol increased spine density in HA-DISC1 HA-DISC1ΔCT expressing cells to a level not statistically different to untreated Ctl level ($F(5,111)=17.63$, $p < 0.001$, Tukey Post Hoc, *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$, one-way ANOVA; n = 16-25 cells per condition from 5 independent cultures). Scale bar = 5 µm.

Figure 3: 17β-estradiol (E2) reduces DISC1 aggregation. (A) Representative images of DIV 26 cortical neurons expressing either HA-DISC1 or HA-DISC1 ΔCT were treated with vehicle or 17β-estradiol (E2) for 30 minutes. Pseudo-color scheme is used to highlight regions where DISC1 clustering is occurring. **(B)** Quantification of DISC1 clustering; both HA-DISC1 or HA-DISC1 ΔCT formed large clusters along dendrites. Treatment with E2 reduced DISC1 Clustering in neurons expressing either HA-DISC1 or HA-DISC1 ΔCT (*, $p < 0.01$ (corrected for multiple comparisons)

Student t-test; n = 12-13 cells per condition from 3 independent cultures). Scale bar = 5 µm.

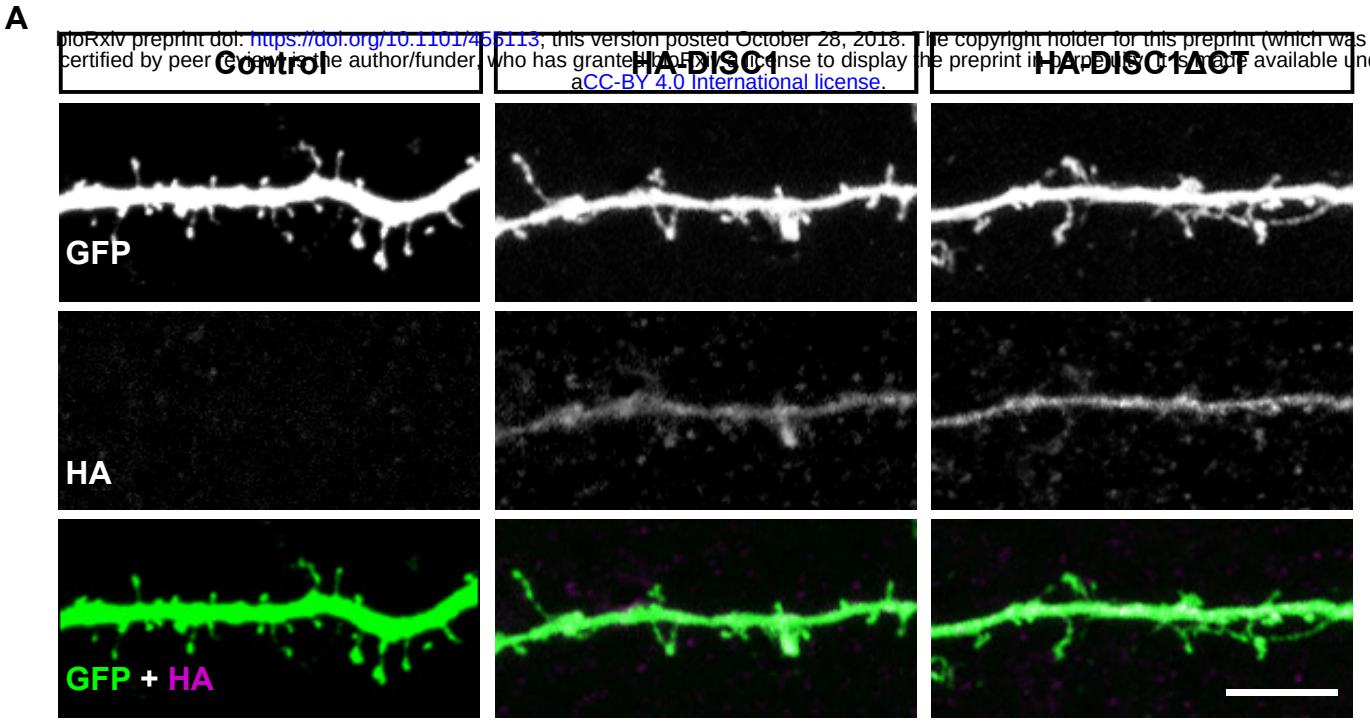
Figure 4: Acute 17β-estradiol (E2) treatment restores dendritic spine density following long term Disc1 knockdown. (A and **B)** Western blot of hEK293 cells expressing HA-Disc1 in presence of control shRNA vector or DISC1-shRNA **(A)**. Quantification of HA-DISC1 expression **(B)**, normalized to β-actin demonstrates that DISC1_shRNA effectivity knockdown exogenous Disc1 as previously reported. **(C)** Representative images of cortical neurons expressing Ctl vector (pGSuper) or shRNA against Disc1 (Disc1_shRNA) for 7 days; neurons were treated with vehicle (Veh) or E2 (10 nM) for 30 minutes. (D) Quantification of dendritic spine linear density (per 10um). As recently reported, expression of Disc1_shRNA for 7 days results in a reduction in spine linear density; as expected 30 minute E2 increases spine density in Clt neurons; intriguingly, E2 treatment for 30 min increased spine density in Disc1_shRNA expressing cells to Clt neuron levels ($F(3,50)=17.38$, $p < 0.001$, Tukey Post Hoc, *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$, one-way ANOVA; n = 12-15 cells per condition from 4 independent cultures).

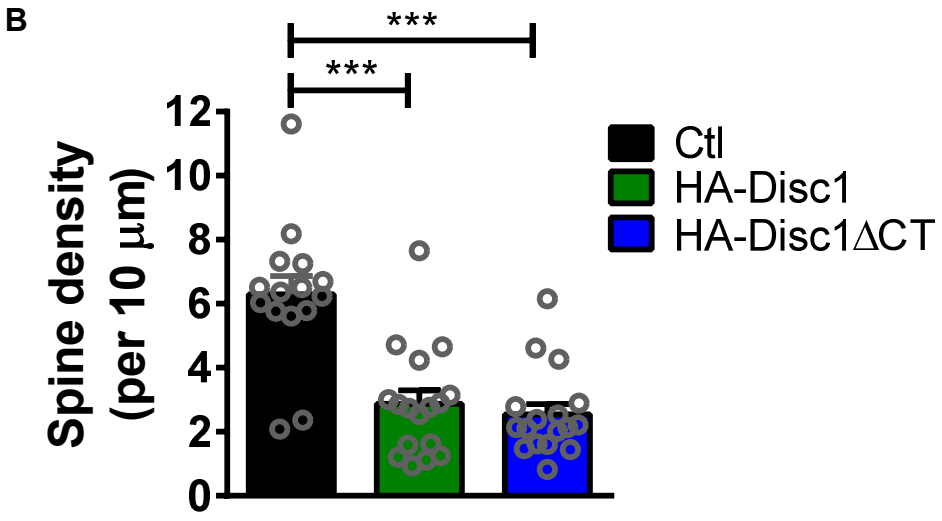
Figure 5: Bi-directional redistribution of DISC1/PSD-95/kalirin7 signalsome by 17β-estradiol (E2). (A) Whole cell lysate, crude synaptosome (P2) and cytosol (S2) fractions of cortical neurons treated with 17β-estradiol (E2) for 0 or 30 minutes analyzed by Western blotting for expression of DISC1, PSD-95 or kalirin-7; β-actin was used as a loading control. E2 has no effect on overall expression levels of DISC1, kalirin-7 or PSD-95 within 30 minutes as seen in whole cell lysate. Treatment

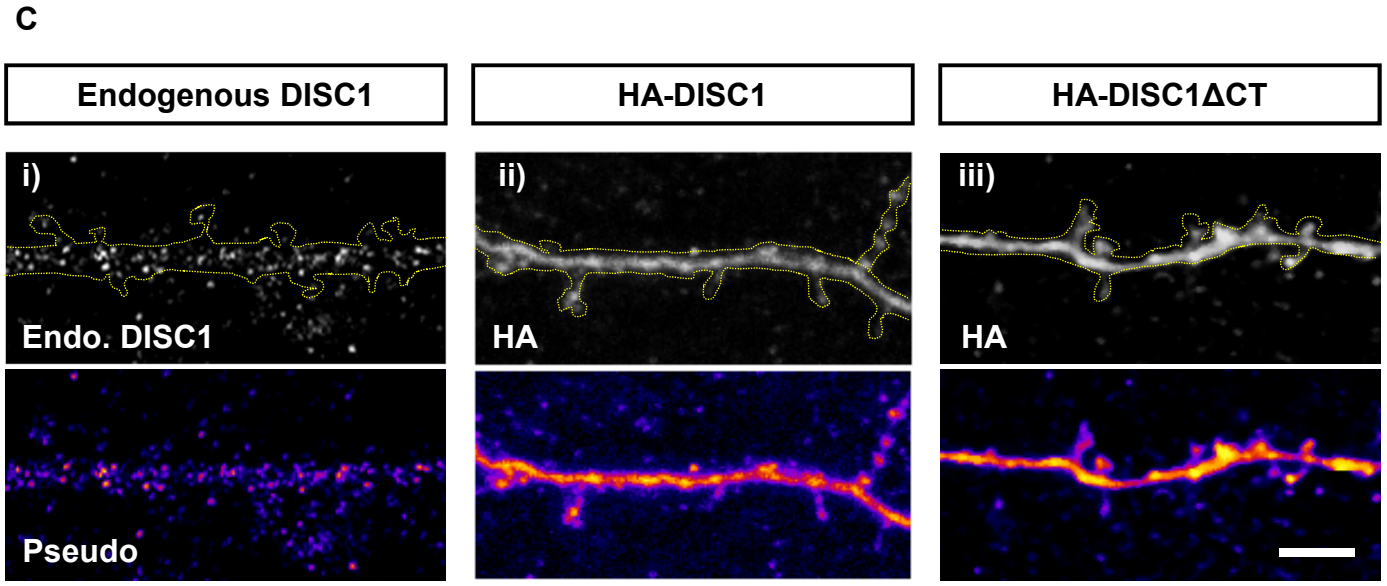
with E2 causes a reduction of DISC1 in crude synaptosome fraction and an increase in cytosolic fraction. E2 treatment resulted in an enrichment of kalirin-7 and PSD-95 within crude synaptosome fraction and concurrent reduction in cytosolic fraction. **(B)** Quantification of DISC1, PSD-95 and kalirin-7 enrichment in crude synaptosome and cytosol fractions (DISC1: ($F(1,8)=24.14$, $p < 0.05$, Bonferroni Post Hoc, *, $p < 0.05$, **, $p < 0.01$, two-way ANOVA; n = 3 independent cultures), PSD-95: ($F(1,8)=8.361$, $p < 0.05$, Bonferroni Post Hoc, *, $p < 0.05$, two-way ANOVA; n = 3 independent cultures), kalirin-7: ($F(1,8)=9.509$, $p < 0.05$, Bonferroni Post Hoc, *, $p < 0.05$, two-way ANOVA; n = 3 independent cultures)). **(C)** GFP-expressing and DISC1 (440) stained cortical neurons treated with E2 for 30 minutes or not. Quantification of DISC1 cluster intensity within spines reveals a reduction in intensity within spines after E2 treatment (*, $p < 0.05$, Student t-test; n = 223-337 spines from 5-6 cells per condition from 3 independent cultures). **(D)** High magnification of areas highlighted by yellow dashed boxes in C; line scan of DISC1 staining within spine heads further demonstrates a redistribution of DISC1 from spines to dendritic shaft after E2 treatment. Scale bar = 5 µm or 1 µm.

Figure 6: PSD-95 and kalirin-7 are enriched at synapses following acute 17β-estradiol (E2) treatment. (A and B) Cortical neurons (DIV 25) fixed before and after treatment with E2 and immunostaining for kalirin-7 and PSD-95. E2 treatment (30 mins) increases kalirin-7 puncta density (black bars). Assessment of number of kalirin-7/PSD-95 co-localized puncta revealed and increase in kalirin-7 and PSD-95 positive puncta (red bars), indicating that kalirin-7 is being targeted to synapses (*, $p < 0.01$ (corrected for multiple comparisons) Student t-test; n = 12-15 cells per condition from 3 independent cultures). **(C)** GFP-expressing and kalirin-7 stained

cortical neurons treated with E2 for 30 minutes or not. Quantification of kalirin-7 cluster intensity within spines reveals an enrichment within spines following treatment (*, $p < 0.05$, Student t-test; n = 270-341 spines from 6 cells per condition from 3 independent cultures). **(D)** High magnification of areas highlighted by yellow dashed boxes in C; line scan of kalirin-7 distribution within spine heads reveals an enrichment of kalirin-7 within spine heads after E2 treatment. Scale bar = 5 µm or 1 µm.

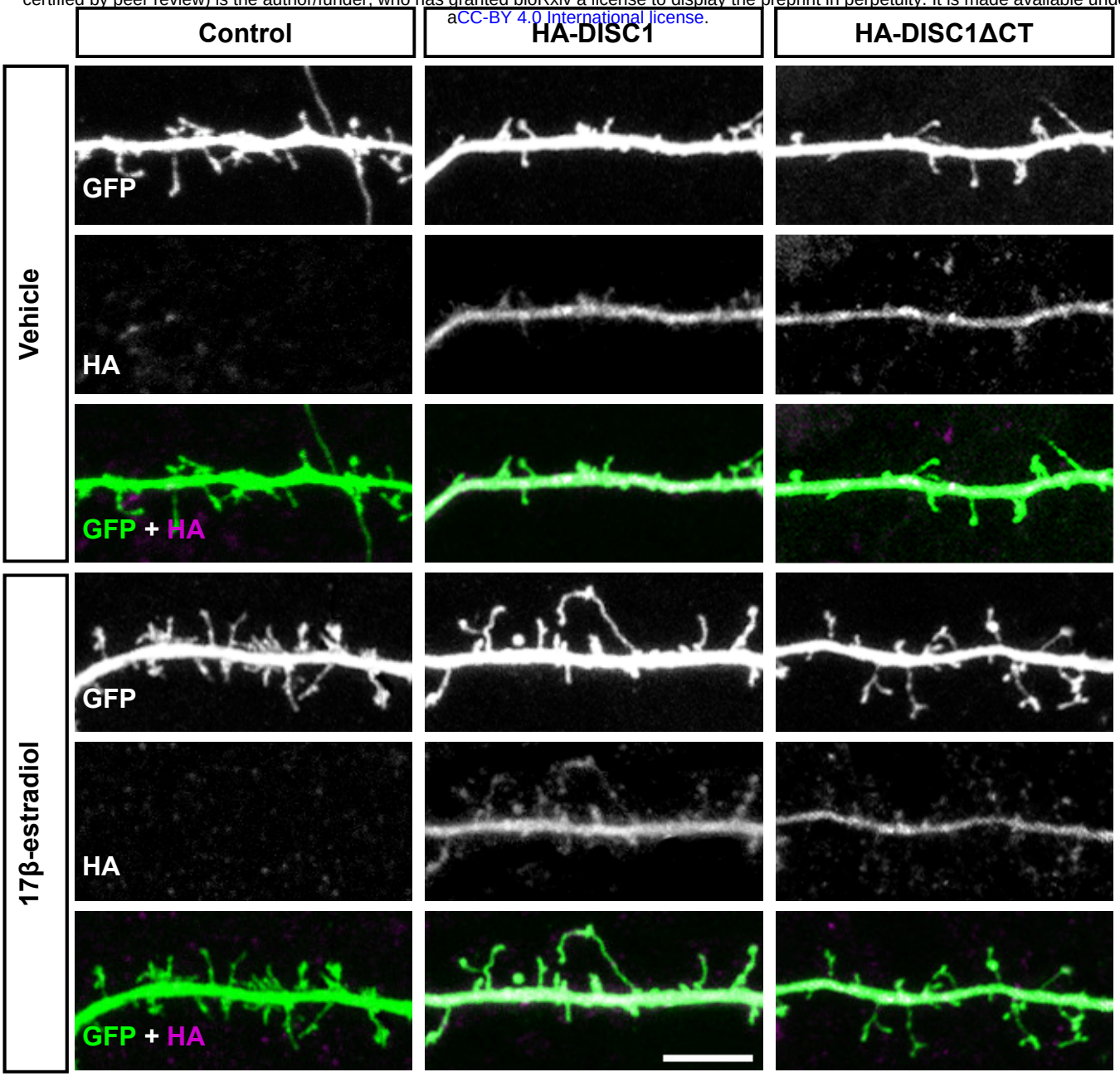






Erli, Palmos et al., Figure 1

A



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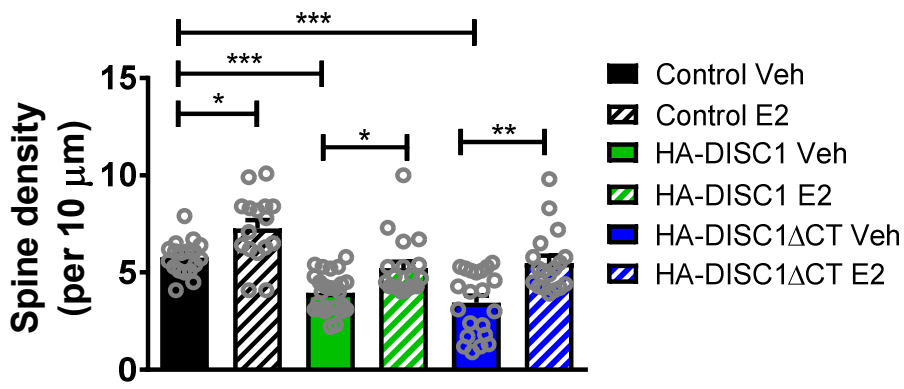
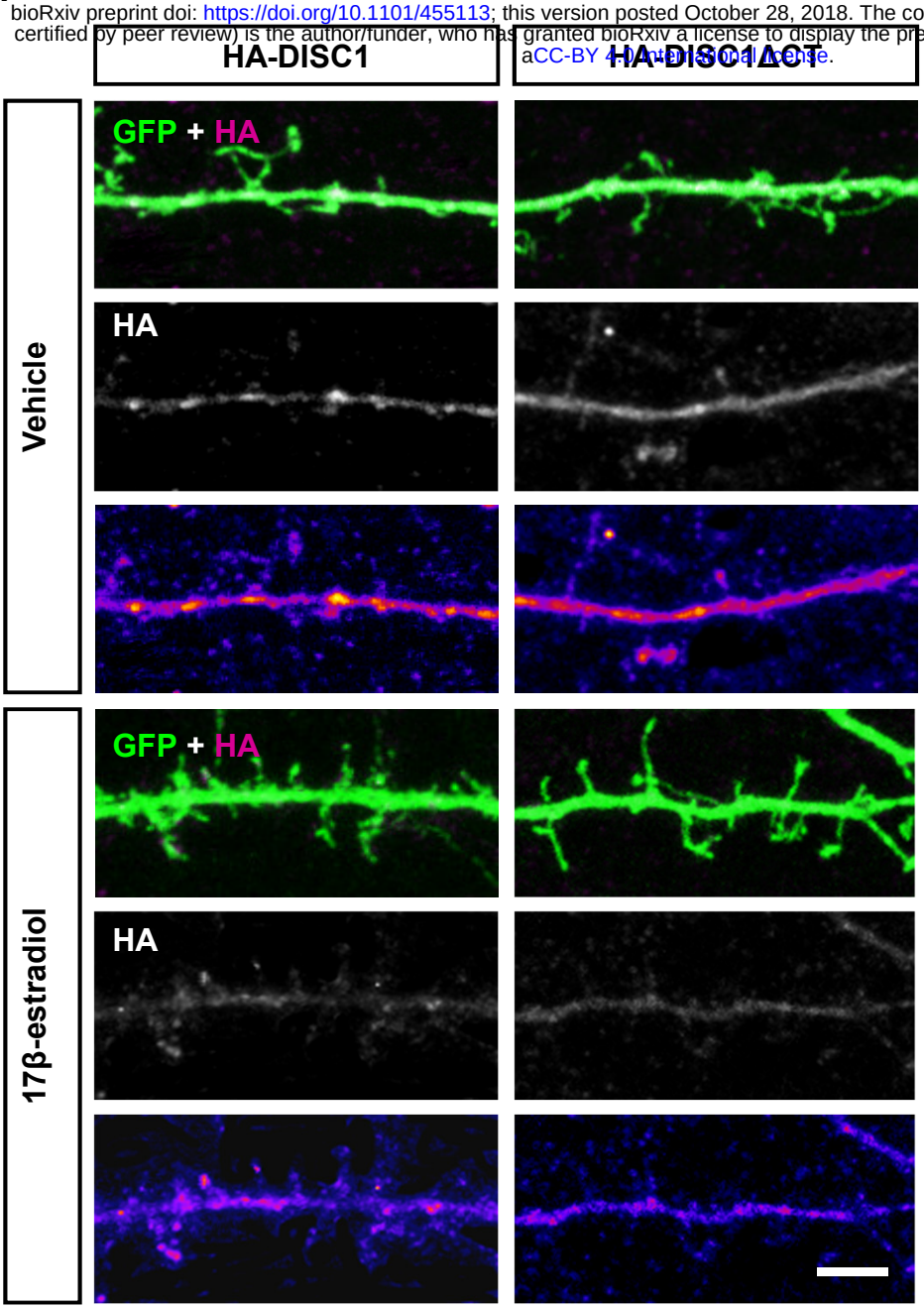


Erli, Palmos et al., Figure 2

A



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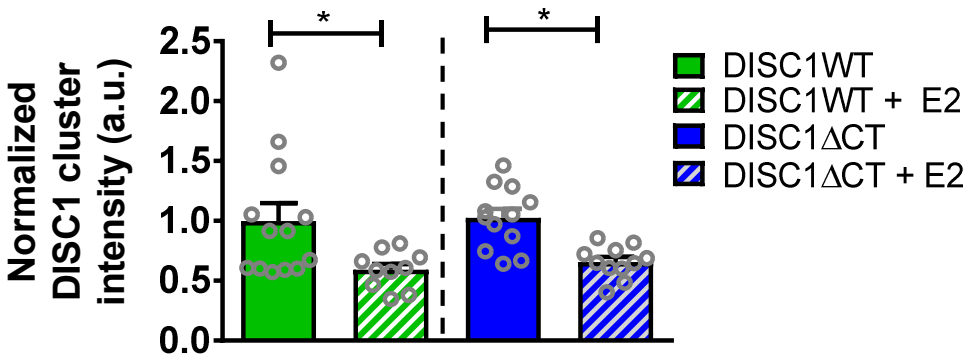
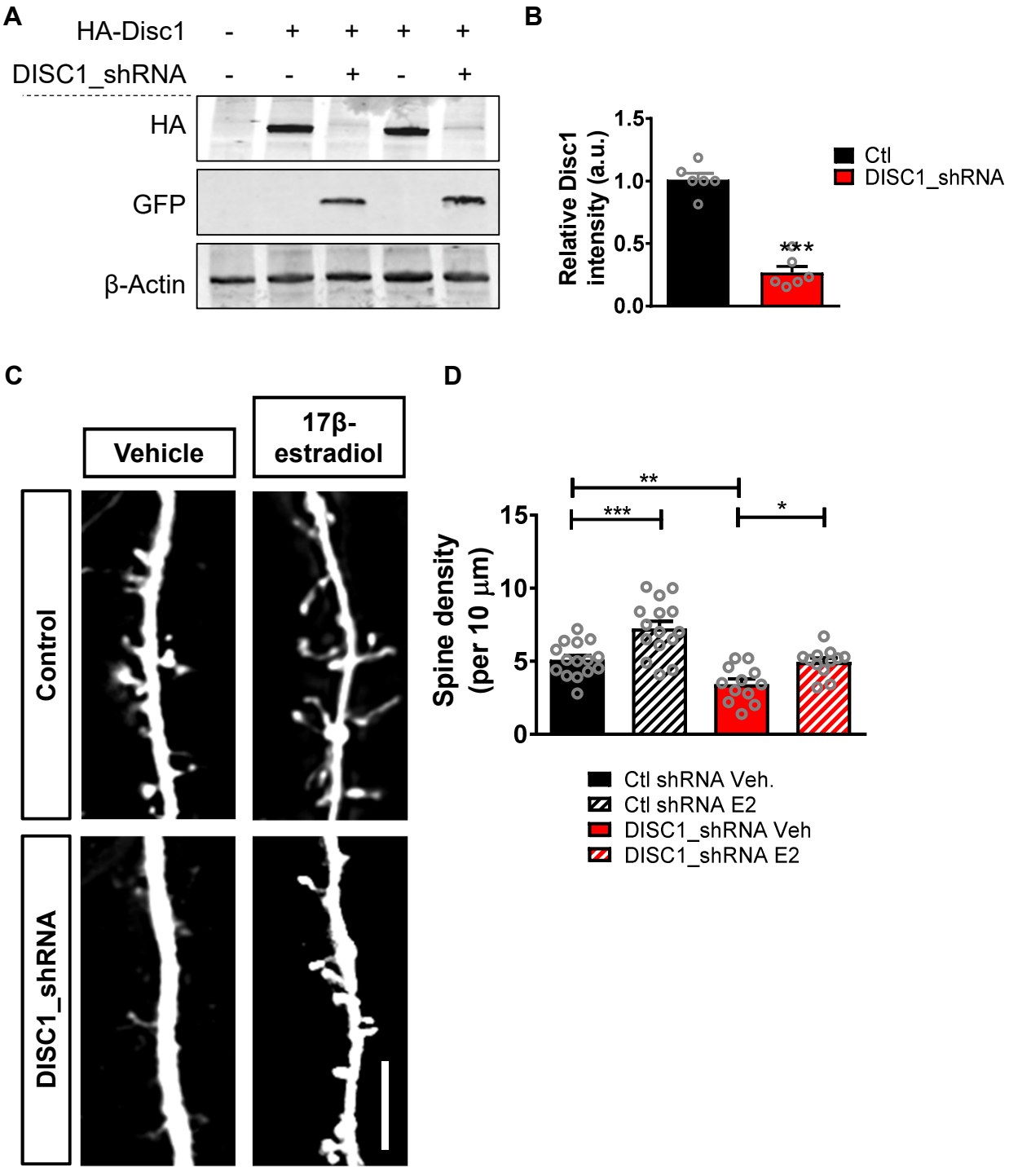
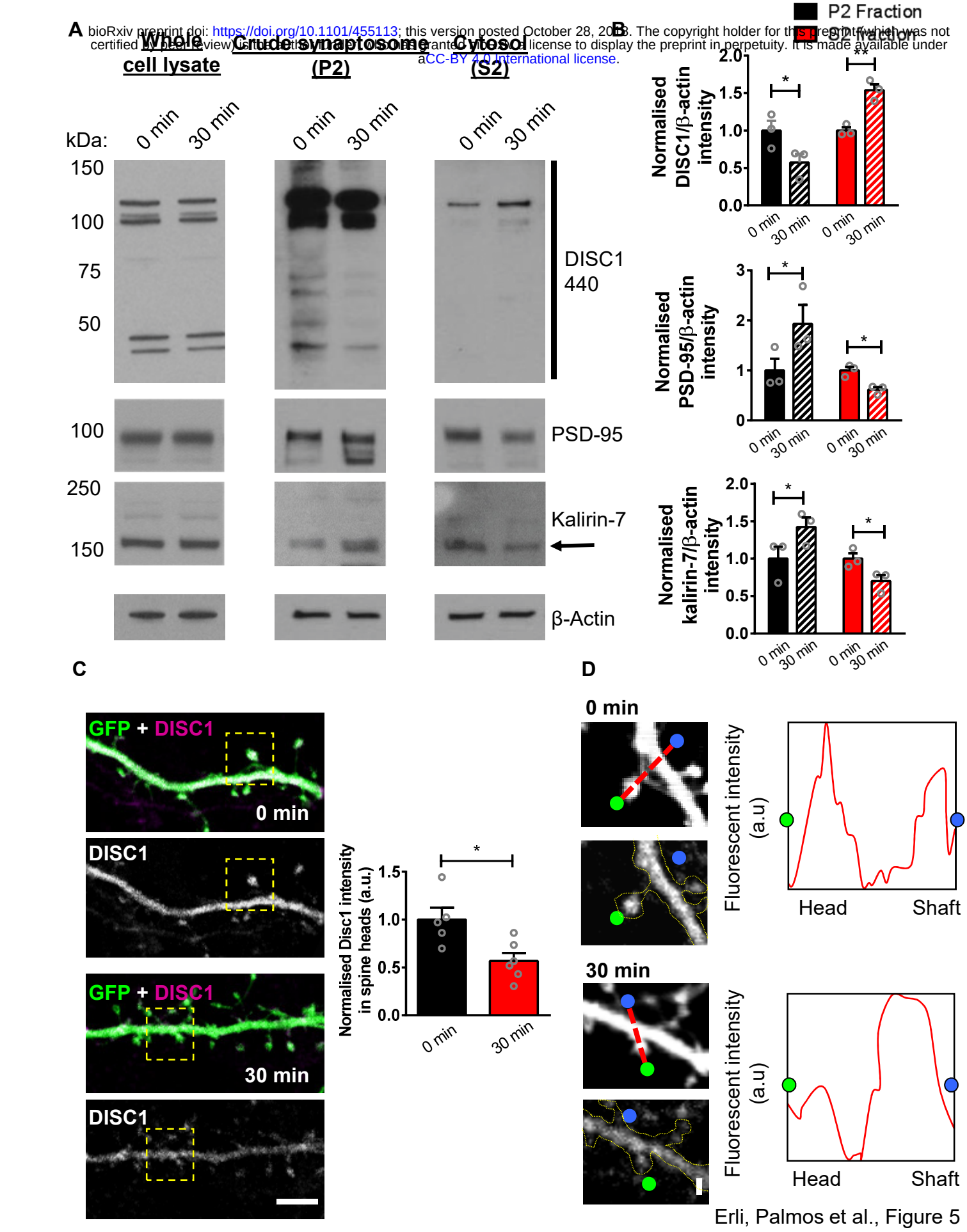


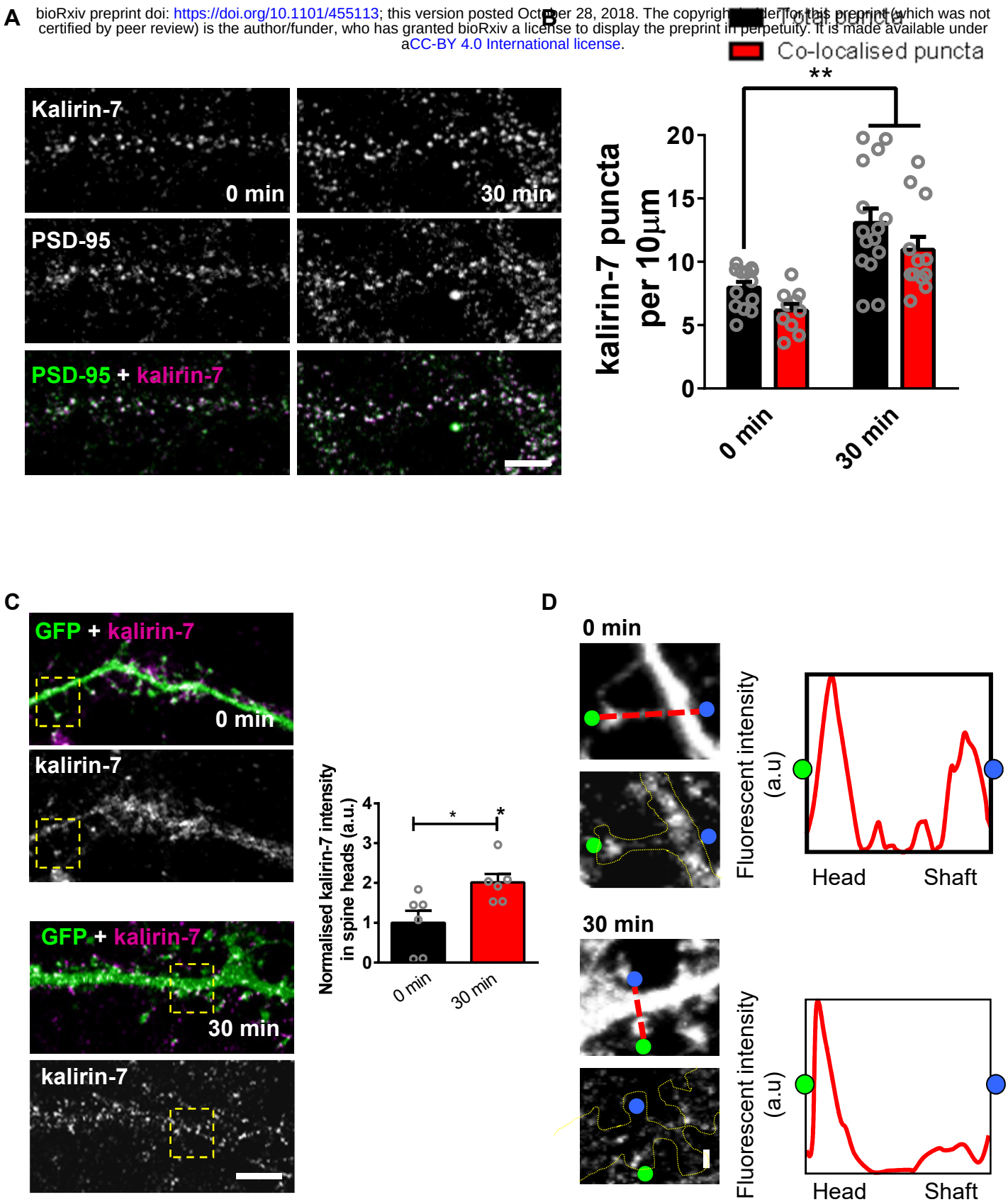
Erli, Palmos et al., Figure 3



Erli, Palmos et al., Figure 4



Erli, Palmos et al., Figure 5



Erli, Palmos et al., Figure 6