Stress, reelin, and the entorhinal cortex: exploring the effect of chronic stress on reelin density in the rat entorhinal cortex and the correlations with cognition, emotion, and inflammation

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We acknowledge and respect the Lək̓ʷəŋən (Songhees and Esquimalt) Peoples on whose territory the university stands, and the Lək̓ʷəŋən and W̱SÁNEĆ Peoples whose historical relationships with the land continue to this day.

Supervisory Committee

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Abstract

Chronic stress is a risk factor for depression, Alzheimer's disease, and other disorders. Reelin is a protein with roles in cortex development, synaptic plasticity, and immune responses. Reelin is dysregulated in the hippocampus, hypothalamus, and other regions in these disorders and others. The entorhinal cortex (EC) plays an important role in episodic and spatial memory: with ties to the hippocampus, reelin expression, and shows alterations in depression. To examine the effect of chronic stress on the reelin positive (reelin$^+$) cells in the EC and the connections to cognitive, emotional, and immune systems, 36 rats underwent a 14 week cyclic chronic stress model of depression. A subset of rats received an injection of reelin on the last day to test whether reelin treatment reverses depression-like behaviour and restores reelin in the EC. Behavioural tests were conducted, and immunohistochemistry was used to stain reelin$^+$ cells. Reelin treatment reversed depression-like behaviour, but no effect of chronic stress on EC reelin$^+$ cell density was observed, suggesting that chronic stress does not affect the levels of reelin$^+$ cells in the EC as it does in other regions such as the dentate gyrus. Correlations were examined between reelin$^+$ cell density, forced swim test immobility, spatial memory, and spleen white pulp (WP) area. There was no relationship between reelin$^+$ cell density and immobility time, however there was a significant correlation between spatial memory and reelin cell density in males. This correlation was disrupted with chronic stress but successfully recovered with a reelin injection, suggesting a sex-specific relationship between spatial memory and reelin levels in the EC. Males showed significant white pulp atrophy and recovery with reelin, but only females showed a significant correlation with reelin$^+$ cell density. This suggests a role of reelin in modulating inflammatory responses, and highlights another sex-specific difference. The disruption by chronic stress in the correlation between cognitive tests and EC reelin$^+$ cells, and the subsequent recovery by a single reelin

injection should be further explored when considering the putative fast-acting antidepressant actions of reelin, and that cognitive symptoms in depression patients aren't properly solved by current antidepressant treatment.

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List of Abbreviations

ACTH	Adrenocorticotropic hormone
AD	Alzheimer's Disease
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ApoER2	Apolipoprotein E receptor 2
BPC	Bipolar cells
CDK5	cyclin dependent kinase 5
CI	Confidence interval
CORT	Corticosterone
CR	Corticosterone/reelin
CREB	cyclic AMP response element binding protein
CRH	Corticotropin releasing hormone
CV	Corticosterone/reelin
DAB	3,3-Diaminobenzidine
Dab1	Disabled 1
dI	De-ionized water
DR	Discrimination ratio
EC	Entorhinal Cortex
EFL	Exploring familiar location
ENL	Exploring novel location
FST	Forced swim test
GABA	γ-Aminobutyric acid
GSK3B	Glycogen synthase kinase-3 beta
HE	Hemotoxin and Eosin
HPA	Hypothalamic-pituitary-adrenal axis
LEC	Lateral entorhinal cortex
LTP	Long term potentiation

MEC	Medial entorhinal cortex
mTOR	mammalian target of rapamycin
NKG2D	Natural killer group 2, member D
NMDAR	N-methyl-D-aspartic acid receptor
OBiPT	Object in place test
PBS	Phosphate buffered saline
PI3K	Phosphoinositide3 kinase
PKB	Protein kinase B
Reelin^{+}	Reelin-positive
S6K1	S6 kinase 1
SEM	Standard error of the mean
SERT	Serotonin transporter
SGZ	Sub-granular zone
TBS	Tris-buffered saline
TRD	Treatment resistant depression
VLDLR	Very low density lipoprotein receptor
VV	Vehicle/Vehicle
WP	White pulp

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Liam, even when you were away sailing for months you always let me talk about the project and everything that I had to get done. Even if you didn't understand what I was talking about, you believed in me and made me feel like I could do anything, and tried to help me in whatever way you could. You're the bestest!!

My dad somehow proofread this while on a sailboat in the middle of the Pacific Ocean, over a thousand miles from any land, in 3.5m waves and rain, and gave me thorough and detailed feedback. Not sure how you managed to do that, but thank you. So last but not least: thank you to both my parents for the endless support and belief in me.

1: Introduction

1.1: Stress and depression

Stress is hormonally mediated by the hypothalamic-pituitary-adrenal (HPA) axis, where a series of feedback loops control the release of corticotropin releasing hormone (CRH), adrenocorticotropic hormone (ACTH), and cortisol, or corticosterone (CORT) in rodents (Checkley, 1996). Chronic stress is characterized by a consistent upregulation of this pathway, with simultaneous downregulation of the components involved in negative feedback (Checkley, 1996). Chronic stress has been shown to be a key risk factor in the development of depression, as well as neurodegenerative diseases such as Alzheimer's Disease (AD) (Anisman & Zacharko, 1982; Bisht et al., 2018; Checkley, 1996; Justice, 2018; Song et al., 2020).

Depression affects millions of people, and can be characterized by a loss of energy, change in sleep, anhedonia, and a depressed mood (Buchwald & Rudick-Davis, 1993; Richards, 2011). The exact cause of depression is not yet known, however there are many factors that are thought to play a role in the development of depression. These involve environmental, genetic, and molecular influences including prior chronic stress and resulting prolonged HPA axis activation (Checkley, 1996; D. N. Klein et al., 2013; Nestler et al., 2002). There are clear sex differences, with almost twice as many females diagnosed as males (Mohammadi et al., 2023; Richards, 2011). One potential molecular cause for depression was thought to be altered monoamines such as serotonin and dopamine (Ly et al., 2018; Stockmeier et al., 2004), however this is still under discussion (Moncrieff et al., 2023). The volume of both the hippocampus and the entorhinal cortex (EC) are decreased in patients with depression, and the synaptic plasticity in the hippocampus is changed (Furtado et al., 2008; Xu et al., 2020). Depression can have effects on cognition such as

memory deficits and a negative processing bias (Kwak et al., 2016), and depression is a risk factor for AD (Byers & Yaffe, 2011; Kwak et al., 2016).

Depression often presents as depressive episodes, and after experiencing one episode there is a high chance of recurrence (Pettit et al., 2006). Recurring episodes can make antidepressants less effective, which can lead to treatment resistant depression (TRD): depression in which the patient does not achieve remission after at least two treatments with antidepressants (Berlim & Turecki, 2007; Fava, 2003). To mimic the episodic pattern of depression, the cyclic CORT model was developed (Lebedeva et al., 2017). This rodent model uses repeated injections of CORT (mimicking chronic stress) followed by a rest period, and then another round of injections to use chronic stress to induce depression-like behavior. Tests of depression such as the forced swim test (FST) consistently show these animals as expressing more depression-like behaviour and depressive phenotypes than control animals (Brummelte & Galea, 2010; Gregus et al., 2005; Johnson et al., 2006; Lebedeva et al., 2017; Lussier et al., 2013).

1.2: Reelin

Reelin is a large extracellular matrix protein that plays a key role in neural development, specifically in the layering of the cortex. In the cortex, reelin is secreted by Cajal-Retzius neurons, neurons also essential for cortical development (Frotscher, 1998; Tissir & Goffinet, 2003). It acts as a signal protein for neuronal migration, where it both assists in forming the scaffolding system of radial glia and is a stop and detach signal for migrating neurons (Curran & D'Arcangelo, 1998; Förster et al., 2002; Hack et al., 2002). Mice lacking reelin display improper neuronal positioning, with dysregulation in forming cortical layers (Curran & D'Arcangelo, 1998; Tissir & Goffinet, 2003). Post-development, Reelin continues to play a role in the adult brain. Reelin has functions in the pre- and post-synapse, and is involved in both synaptic plasticity, where it assists in

enhancing long-term potentiation (LTP); and spine formation and maturation, where it works to increase both the number and strength of connections, and to increase the density of spines (Wasser & Herz, 2017).

In the hippocampus and cortical regions Reelin is primarily secreted by γ-aminobutyric acid (GABA)ergic interneurons, whereas in the cerebellum it is secreted by glutamatergic neurons and in the EC by layer II pyramidal neurons (Kobro-Flatmoen et al., 2016; Pesold et al., 1998). Reelin has two processing sites, resulting in a C-fragment, N-fragment, and central fragment. The central fragment is primarily what binds to the reelin receptors: very low density lipoprotein receptor (VLDLR) and apolipoprotein E receptor 2 (ApoER2) (Jossin, 2020).

The receptors trigger phosphorylation of Disabled 1 (Dab1), causing Src family tyrosine kinases (SFKs, e.g. Fyn) phosphorylation of N-methyl-D-aspartic acid receptor (NMDAR) (Figure 1) (Lussier et al., 2016). NMDAR activation results in an influx of calcium, leading to α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) insertion and activation, as well as activation of cyclic AMP response element binding protein (CREB) (Lussier et al., 2016). This leads to an increase in synaptic plasticity (Lussier et al., 2016). Phosphoinositide3 kinase (PI3K) interacts with Dab1 to activate, which then in turn causes protein kinase B (PKB) phosphorylation (Jossin, 2020). PKB goes on to inhibit Glycogen synthase kinase β (GSK3B), leading to inhibition of tau phosphorylation, as well as activating mammalian target of rapamycin (mTOR) and S6 kinase 1 (S6K1), leading to synaptic development and dendritic growth (Jossin, 2020). Tau phosphorylation can also be achieved through the protein p35, which is converted to p25 and activates cyclic dependent kinase 5 (CDK5) (Lussier et al., 2016).

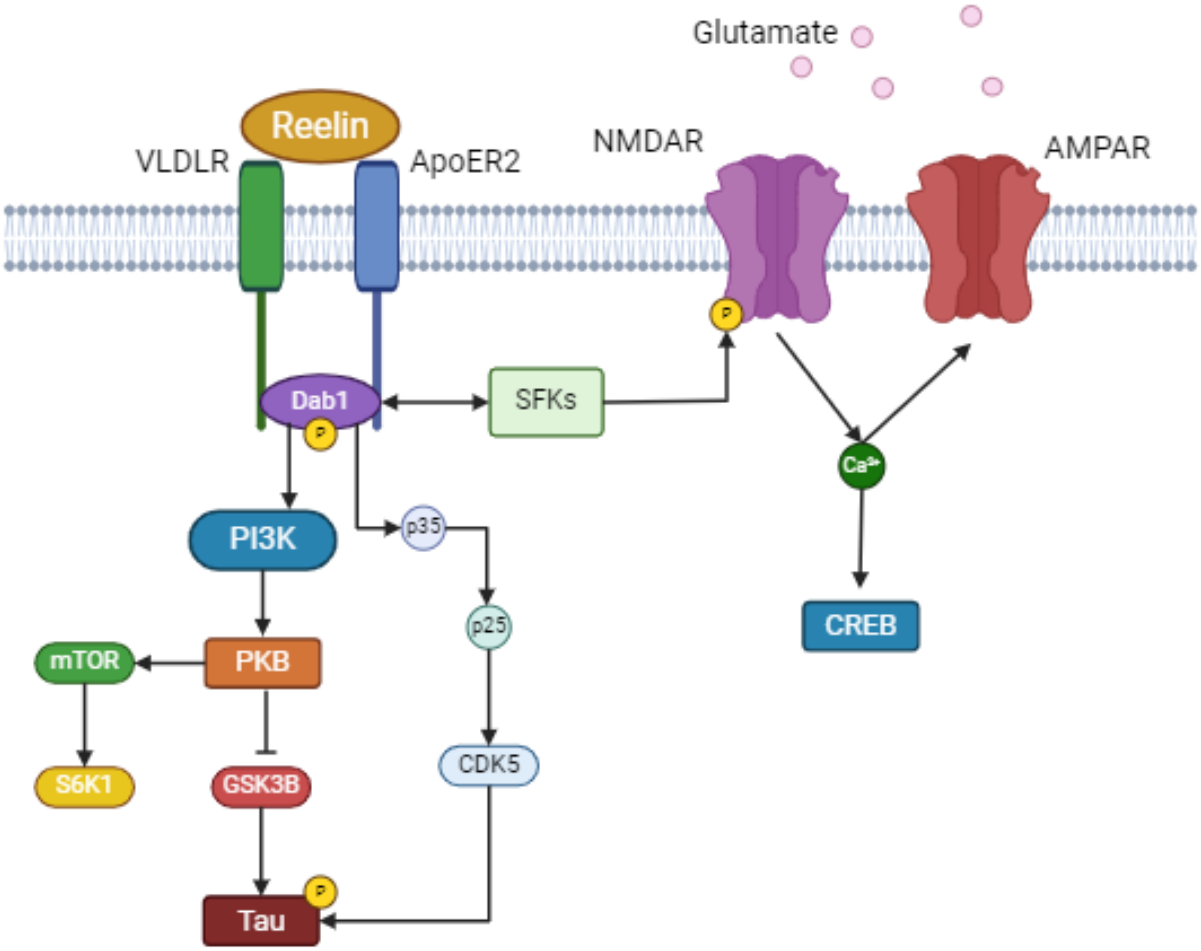

Figure 1. Reelin transduction pathway. Reelin binds to lipoprotein receptors VLDLR and ApoER2, which phosphorylate Dab1, thus activating PI3K and PKB. PKB inhibits GSK3B, supressing the phosphorylation of tau. Dab1 can activate p35, resulting in conversion to p25 and CDK5 activation. PKB activates mTOR, leading to S6K1 activation. Dab1 also activates SFKs, which phosphorylate NMDA receptors, leading to an influx of Ca^{2+} and activation of both CREB and AMPA receptors. Figure created in Biorender.com, adapted from Lussier et al. (2016).

Reelin can be found throughout much of the body. It is primarily found in the brain: in endothelial cells ; layer II of the EC; the dentate gyrus, hippocampal fissure, and stratum oriens in the hippocampus; and external granule layer of the cerebellum, and other cortical areas (Perez-Costas et al., 2015; Pesold et al., 1998). The liver is a key region in the production of reelin, where it can be found in hepatic stellate cells. This is potentially a source for the reelin found circulating in peripheral blood (Kobold et al., 2002; Smalheiser et al., 2000).

Reelin is dysregulated in a multitude of disorders. In the EC and hippocampus, reelin depletion can be an early marker for AD (Chin et al., 2007; Herring et al., 2012). In depression, reelin is dysregulated in many regions of the hippocampus including the sub-granular zone (SGZ) of the dentate gyrus, hilar areas, and CA4 (Fatemi et al., 2000; Reive et al., 2024). These same hippocampal areas, as well as regions of the prefrontal cortex, show reductions of reelin in schizophrenia and bipolar disorder (Fatemi et al., 2000; Guidotti et al., 2000; Impagnatiello et al., 1998). Recent research has been exploring the therapeutic properties of reelin in acting as an antidepressant following chronic stress models of depression. Reelin injections have recovered levels of reelin in the SGZ, reduced depression-like behaviour, recovered spleen white pulp (WP) atrophy, partially recovered neurogenesis in the hippocampus, and rescued deficits in spatial memory and long-term potentiation (Allen et al., 2022; Brymer et al., 2020; Johnston et al., 2023; Reive et al., 2023, 2024). When reelin functions as an antidepressant it does so as a fast-acting one, with a downstream pathway likely that of ketamine, and involving NMDA and AMPA receptors (Brymer et al., 2020; Johnston et al., 2023).

1.3: The entorhinal cortex

The EC is a region important for both spatial and episodic memory, and is considered the gateway between the hippocampus and the complex cortical regions. Located near the rhinal sulcus, it can be divided up into the medial EC (MEC) and lateral EC (LEC), with similarities and differences between morphologies, functions, and connections of the two.

Like other cortical regions, the EC has six layers each with slightly different functions and morphology. Layers III-VI are much more similar than layers I and II. As a brief overview of the characteristics and similarities between the layers: horizontal cells can be found in layers I, V, VI; multi-polar neurons are in layers I, III, V, VI, pyramidal neurons are in all layers but layer I, and

bipolar cells (BPC) are in layers III, V (Canto et al., 2008). Fan cells can be found in layer II of the LEC and stellate cells in layer II of the MEC, however these have similar functions (Canto et al., 2008).

The focus of this paper is on layer II of the LEC. As previously stated, this region primarily contains fan cells and pyramidal neurons (Canto et al., 2008). Fan cells are crucial for episodic memory, specifically for forming associations between objects, contexts, and place (Vandrey et al., 2020). The pyramidal neurons are excitatory (glutamatergic), and key in connecting the EC with the hippocampus. They project from layer II to regions in the hippocampus. Layer II of the LEC also expresses the most reelin, in fact almost all of the cells in this layer are reelin-positive ($reelin^{+}$) notably including the excitatory pyramidal neurons that project to the dentate gyrus. (Pesold et al., 1998; Ramos-Moreno et al., 2006). This is a difference between the LEC and the MEC, where the MEC expressed much lower levels of reelin compared to the LEC.

The EC primarily projects to the hippocampal regions. Layer II excitatory pyramidal neurons primarily project to the dentate gyrus and CA2/CA3 regions (Figure 2), whereas neurons in layer III project to CA1 and the subiculum (Witter, 2009). This projection to the hippocampal regions occurs through the perforant pathway. In this pathway, the axons leave layers II and III of the EC, converge in the angular bundle, and then continue on to the hippocampal regions (Walton, 2012). The EC also projects to cortical regions including the parahippocampal and perirhinal cortices and olfactory structures (Canto et al., 2008; Witter, 2009). It acts as a translator or compressor of information, receiving input from sensory regions of the 6-layered cortex and projecting to the 3-layered hippocampal integration regions. It receives input from a wide variety of regions, primarily from the parahippocampal and perirhinal cortices but also from the olfactory bulb, piriform cortex, and parietal cortex (Canto et al., 2008; Witter, 2009). Olfactory and

perirhinal inputs to the more superficial layers I and II, whereas the parietal cortex inputs to layers I and VI (Canto et al., 2008).

Overall, the EC is necessary for spatial memory. The MEC contains grid cells, which use synaptic plasticity to form a map of novel locations (Malone et al., 2024). Many of the excitatory neurons in the LEC are spatially modulated, and others are involved in object coding (Huang et al., 2023). The spatially sensitive neurons create a map along the antero-posterior axis that can be altered by contextual changes. While the LEC codes for both objects and context separately, it also provides integration of these before projection to the hippocampus (Huang et al., 2023).

Alterations in the EC are seen in multiple disorders. This is the first region showing neurodegeneration in Alzheimer's Disease (AD) (Braak et al., 1993). Additionally in AD patients, a thinner EC is correlated with decreased cognitive abilities (Velayudhan et al., 2013). Prasad et al. (2004) found significantly reduced EC volume compared to control participants in patients with a variety of both schizophrenic and non-schizophrenic disorders, as well as in patients with non-delusional psychotic disorders. Increased neural activation is seen in the EC in restraint stress, suggesting involvement in processive stress responses: or stressors that involve cognitive processing of sensory information (Umegaki et al., 2003). In depression, multiple molecular pathways in the EC are altered, including neurotransmitters such as glutamate, serotonin (and SERT, the serotonin transporter), and norepinephrine being upregulated (Chen et al., 2020). The overall volume of the EC is also reduced in female patients with treatment-resistant depression (Furtado et al., 2008). Both the anatomical and metabolic changes seen support the involvement of the EC in depression.

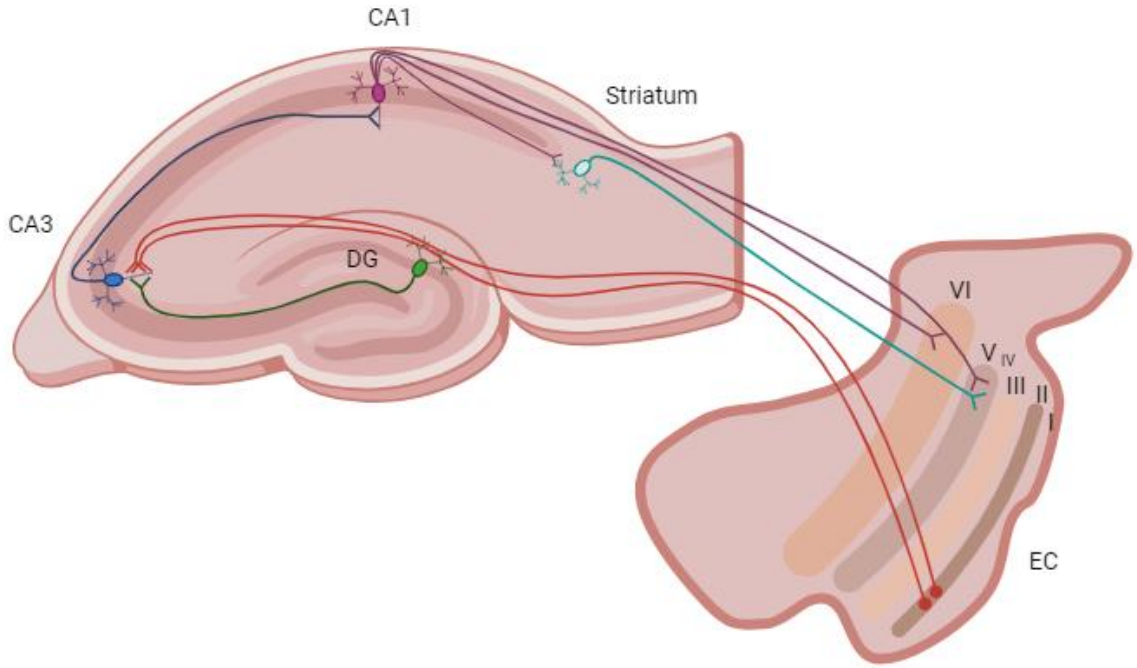

Figure 2. Connections from the EC to the hippocampus. Projections from layer II of the EC project to the dentate gyrus (DG) and CA3 regions, from which projections continue along the hippocampal circuitry to CA1 and the striatum. Figure created at Biorender.com, adapted from Patten et al. (2015).

1.4: The spleen

The spleen is important for filtering waste products out of our blood as part of the immune system. It is made up of red pulp, white pulp (WP), and the marginal zone: each with slightly different functions. The red pulp is key for storing blood cells, recycling iron, and filtration of arterial blood with the use of macrophages (Mebius & Kraal, 2005; Wei et al., 2022). The marginal zone is a transition region through which cells reach the WP from the blood, as well as producing necessary cytokines for inflammatory responses (den Haan & Kraal, 2012; Mebius & Kraal, 2005; Wei et al., 2022). The WP contains both T cells and B cells, which are activated in immune responses. It works alongside the lymphatic system, and contains chemokines which organize the T and B cells into separate zones (Mebius & Kraal, 2005; Wei et al., 2022).

Stress and depression both affect the spleen. Stress can result in enlargement of the spleen, can alter cytokine levels, and can result in increasing macrophage accumulation in the brain

(Bronte & Pittet, 2013; Wei et al., 2022; Zhang et al., 2021). Chronic stress can also result in atrophy of the WP (Reive et al., 2023). In depression, dysregulation of the spleen can be involved in neuroinflammation. The cytotoxic receptor NKG2D (natural killer group 2, member D) is increased in the spleen, thus increasing the stimulation of downstream cytokines (Wensveen et al., 2018; Zhang et al., 2021). Additionally, inflammation (part of the immune response), is seen in both stress and depression (Lee & Giuliani, 2019; Liu et al., 2017).

1.5: Research questions

Stress, reelin, and the EC are connected directly and indirectly. For example: stress dysregulates reelin in the hippocampus, and the EC is highly connected to the hippocampus, and the key projections express reelin. Additionally, stress is a risk factor for AD, which shows alterations in both reelin and the EC. However, it is not yet known how reelin in the EC is affected by chronic stress or how this connects to cognition, emotion, or inflammation. This project uses the cyclic corticosterone model of chronic stress to examine the effect of chronic stress on reelin levels in the EC, on cognition, emotion, and spleen atrophy, as well as the therapeutic properties of reelin. I quantified reelin$^+$ cell density in the EC, spatial memory, depression-like behaviour, and spleen WP area to examine alterations and correlations between reelin in the EC and cognition, emotion, and inflammation following chronic stress and a single reelin injection. I hypothesize that:

1. Reelin$^+$ cell density will be altered in the EC following chronic stress.
2. Reelin$^+$ cell density will be recovered in the EC following a single reelin injection.
3. Reelin$^+$ cell density in the EC will correlate with the observed cognition abilities.
4. Reelin$^+$ cell density in the EC will correlate with the observed depression-like behaviour.
5. Reelin$^+$ cell density in the EC will correlate with the observed spleen WP area.

2: Methods

2.1: Animals

The animals used were 36 Long Evans rats, consisting of 18 male and 18 female rats. Rats were 12 weeks old at the experimental onset and weighed approximately 300g (±100g). All rats were housed alone, and all received a constant temperature and 12:12 hour light/dark cycle. To reduce the extra stress from handling, the rats were handled once per day for a week leading up to the start of the experiment. All procedures followed the regulations outlined by the University of Victoria Committee on Animal Care and the Canadian Council on Animal Care. For more details around breeding, see Reive et al. (2024).

2.2: Cyclic corticosterone model

The rats were randomly assigned to three different treatment groups, with 6 male and 6 female rats in each group. Researchers from the Caruncho lab performed 2.6 cycles of the cyclic corticosterone (CORT) model on the rats, as outlined by (Lebedeva et al., 2017). This model is designed to induce chronic stress in a way that mimics depressive episodes. One cycle consists of 21 days of CORT injections (20mg/kg) or vehicle injections, one injection per day, followed by a 21-day rest period. The last set of injections lasted two weeks instead of the full three. In addition to CORT, the solutions consisted of 0.9% physiological saline (pH = 7.4) and 2% Tween-80. To reduce stress from the injections, each rat received an appetitive stimulus after the injection and was not restrained throughout the process.

On the final day of CORT injections, reelin or vehicle injections were given as an injection in the tail vein 1hr post-CORT injection. Figure 3 details the experimental timeline. Reelin injections contained 3μg of recombinant reelin in 0.1% phosphate-buffered saline (PBS).

The three treatment groups are as follows: Treatment group 1 (control: vehicle/vehicle, "VV") received vehicle injections at the same time the other groups received both CORT and reelin injections. Group 2 (CORT: CORT/vehicle, "CV") received CORT injections as per the cyclic CORT model and vehicle injections instead of reelin. Group 3 (reelin: CORT/reelin, "CR") received both the CORT injections and the reelin injection. Figure 4a outlines the three groups.

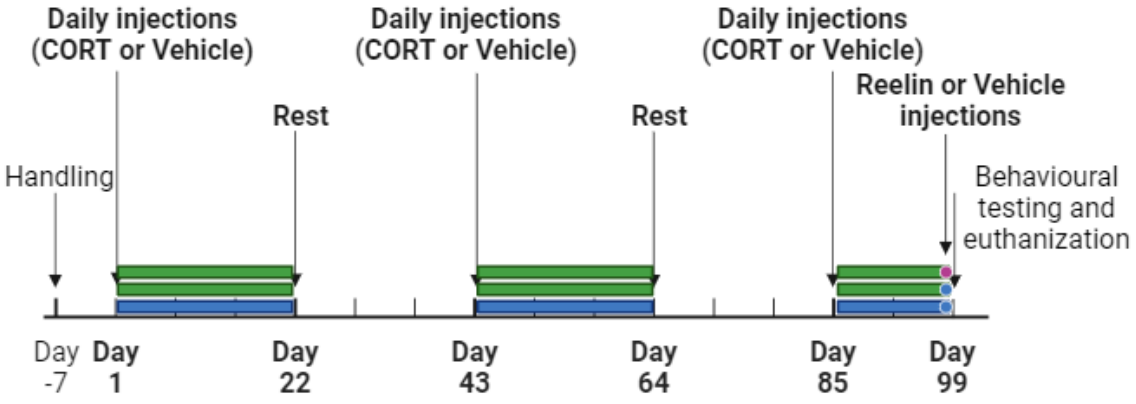

Figure 3. Timeline of experimental procedure: 2.6 cycles of CORT injections. Daily injections are denoted with a solid bar. Vehicle injections = blue, CORT = green. Single reelin injections denoted with a circle overlapping the last day of CORT or vehicle injections, and are either vehicle (blue) or reelin (pink). Figure created at biorender.com.

2.3: Behavioural testing

2.3.1: Object in place test

The object in place test (OBiPT) was used as a measure of spatial memory (Howland et al., 2012). Rats began with training, where they were put in a 65cm^2 arena. This arena had a light blue floor and 60cm high black walls. Four different objects were placed with the rat in the arena, each object 10cm from a different corner. The rats remained in the arena for 10min to become familiar with the layout of the objects. An hour later the positions of two objects were switched, and the rat was placed in the arena for two minutes (Figure 4b). Scoring consisted of duration of object exploration, which was considered to be whenever the nose of the rat went within 2cm of the

object, and was performed while blind to condition. The aim of this test is to compare the duration of exploration of the objects that switched positions (time exploring novel locations: ENL) with the exploration of the original objects (time exploring familiar locations: EFL). This comparison was calculated as a discrimination ratio (DR), with the equation: DR = (ENL - EFL) ÷ (ENL + EFL). A DR of 0 is considered to be chance, or equal time exploration of both locations. For a group of rats to be interpreted to have intact spatial memory, the mean needs to be significantly above 0.

2.3.2: Forced swim test

Researchers from the Caruncho lab performed behavioural testing the day following the final injections (CORT and Reelin). The FST was used to quantify depressive-like behaviour (Can et al., 2012; Porsolt et al., 1977). For this test, rats are placed in a tank of water (30cm deep, approximately 27°C) for ten minutes (Figure 4c). Despair-like behaviour was defined as immobility: periods when the rat stopped struggling to escape the container and instead was swimming only enough to keep the rat's nose above water. This was manually scored for the final five minutes only, because this is when the highest immobility often occurs. The researcher was blind to condition while scoring.

2.4: Tissue collection and staining

Researchers from the Caruncho lab deeply anaesthetized the rats using 5% isoflurane. They then performed a transcardial perfusion, first with physiological saline (0.9%, pH 7.4) and then with paraformaldehyde (4%, pH 7.4). The rats were dissected to collect the brain and spleen. Tissue was placed into 4% paraformaldehyde for one day, then into 10% sucrose in PBS, and lastly a solution of 30% sucrose and 0.1% sodium azide in PBS. The tissue was then frozen in cryoprotectant at -20°C, cryo-sectioned at 20μm with a Leica cryostat, and re-frozen as free-

floating tissue until needed. The brain regions collected were the anterior hippocampus to the posterior entorhinal cortex.

To quantify the density of reelin positive cells in the entorhinal cortex, I performed immunohistochemistry on the free-floating tissue sections (Figure 4d). I began by rinsing the tissue in tris-buffered saline (TBS, 0.1M used throughout). The tissue was blocked to prevent unspecific protein binding, using normal goat serum (15%), Triton X-100 (0.5%), and bovine serum albumin (1%) in TBS. The primary antibody used was mouse anti-Reelin (Millipore, MAB5364) at a concentration of 1:1000, and was incubated for 48 hours at 4°C. Post incubation, I rinsed the tissue with TBS. The next step was incubating in the secondary antibody for two hours. The secondary was biotinylated horse anti-mouse at a concentration of 1:500 (Vector laboratories, BA2001) in block. The tissue was rinsed in TBS and then incubated for 1hr in avidin biotin complex (ABC) at a concentration of 1:500 (Vector Laboratories). The tissue labelling was completed by rinsing in TBS and transferring to 0.02% DAB and 0.0078% H_2O_2. I rinsed the tissue in TBS one last time and then mounted it on Superfrost Plus Microscope glass slides. I used an ethanol serial dehydration prior to cover slipping with Permount (Fischer Scientific, SP15-500) to finish the preparation for microscopy. This procedure resulted in cells expressing reelin becoming a dark brown colour.

To quantify the percentage of white pulp area in the spleens the tissue was mounted on Superfrost Plus Microscope glass slides and stained with hematoxylin and eosin (HE). This process began by fixing the tissue in 70% ethanol and rinsing with deinonized water (dI). The tissue was stained with hematoxylin for one minute. Hematoxylin stains nucleic acids a purple colour (Fischer et al., 2008) The tissue was rinsed with dI, immersed in 0.2% sodium bicarbonate and dI, briefly immersed in acidic ethanol (pH 1.1-1.4), and rinsed in dI again. The tissue was stained with

alcoholic Eosin Y for two minutes. Eosin Y stains the cytoplasm pink (Fischer et al., 2008). The slides were completed by ethanol dehydration, xylene exposure, and cover slipping. This procedure resulted in the white pulp being stained purple because of increased cell density while the remainder of the tissue was stained pink.

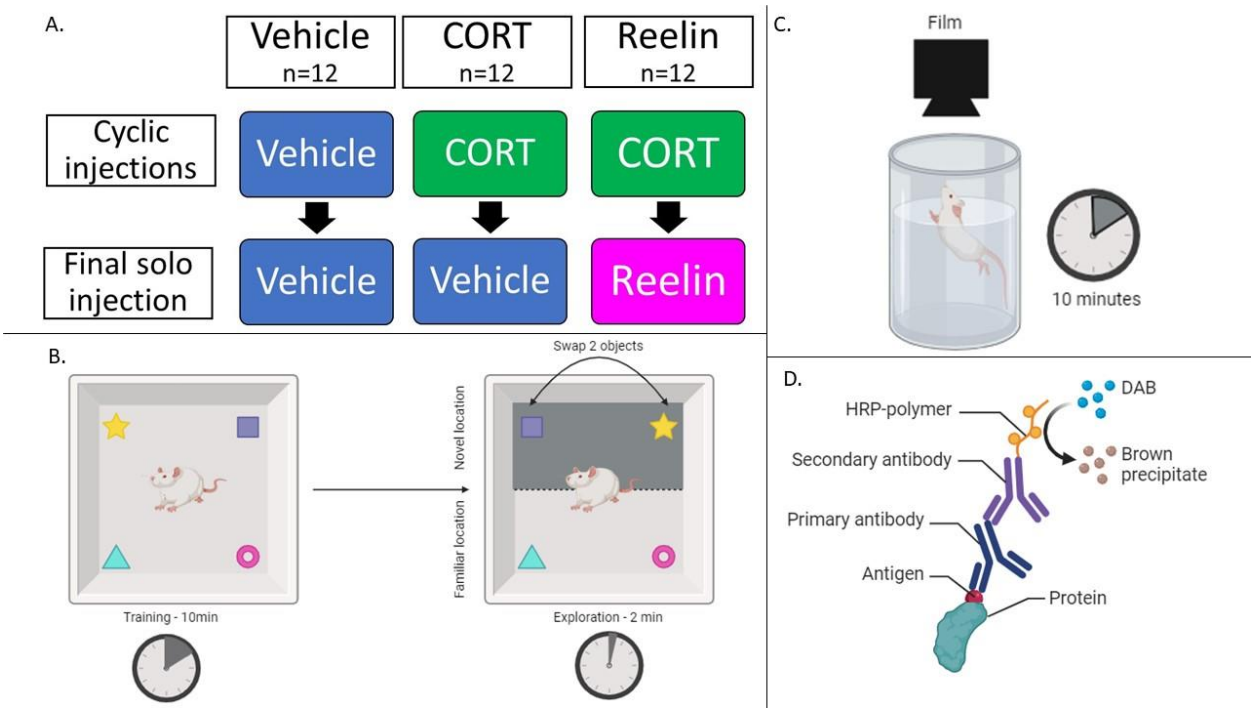

Figure 4. Experimental methods overview. A. Three experimental conditions: VV, CV, or CR. First row corresponds to the injections received during the cyclic CORT model, second row corresponds to injection received on the final day. Each condition had 12 rats (6 male and 6 female). B. Diagram of the OBiPT. The rat is placed in the arena alongside 4 objects for 10 min. 1hr later, two objects are swapped, and the rat is placed in the arena for 2 minutes. The dark grey shading marks the region considered a "novel location", and is purely for design purposes. Design is not to scale. C. Diagram of the FST, where the rat is placed in a tank for 10 minutes. The rat is filmed and is scored later while blind to condition. D. Schematic of immunohistochemistry process used.

2.5: Tissue analysis

I imaged the entorhinal cortex using brightfield microscopy at 63x magnification with oil immersion using a Zeiss microscope. I manually counted the cell density by counting four sections per animal, with five boxes per section, while blind to the condition. Each box was 75µm by 75µm, for a total of 0.1125mm^2 counted per animal. I manually placed each box and counted the total

number of cells within (with inclusion/exclusion bounds in place: Figure 5). The criteria for a box to be placed were that 1. It was centered over the entorhinal cortex, 2. There were a minimum of 2 cells in the box, and 3. There was no overlap with prior sections. Any cell touching the inclusion boundary (top/right) was counted, and any cell touching the exclusion boundary (bottom/left) was not. This prevented bias against whether or not a cell on a border was considered inside the box.

Spleens were imaged using the same Zeiss microscope, at 2.5x magnification. To isolate the hematoxylin from the stains, ImageJ colour thresholding was used. This converts the images to binary black and white, and allows the hematoxylin, and thus white pulp area to be quantified (See Appendix A: Figure 6i). The data collected was the percentage of surface area that white pulp occupied. Researchers analyzed three sections per subject, each section being at least 60µm apart, while blind to conditions.

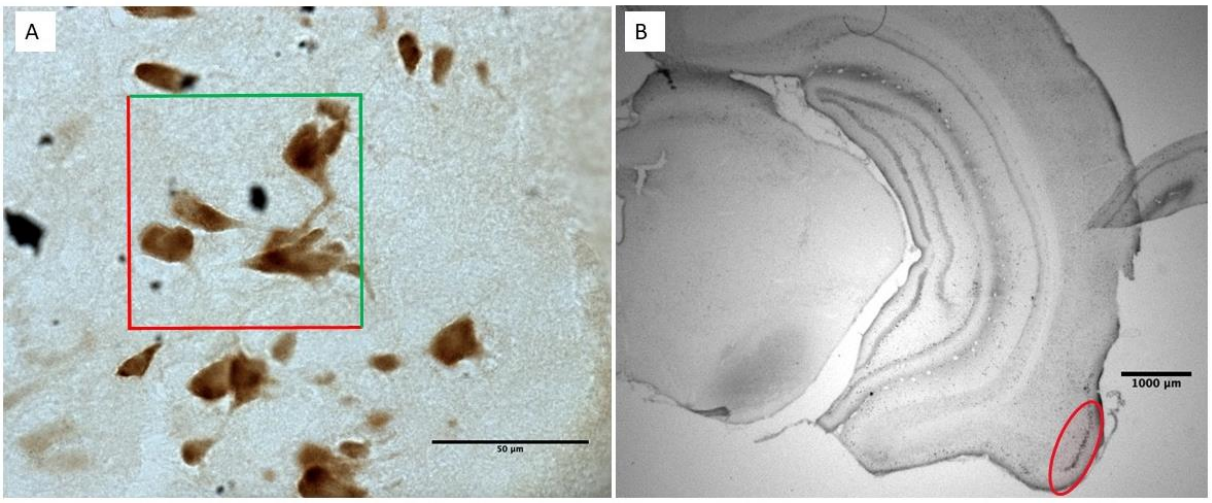

Figure 5. Counting area. A. 75x75µm box used for counts with inclusion areas (green, top right) and exclusion areas (red, bottom left) marked. Dark brown cells are reelin^{+}. Scale bar = 50µm. B. Location of the MEC, circled in red. Scale bar = 1mm.

2.6: Statistics

All statistical analysis were run with the use of Prism 10 software (version 10.0.2). To compare the density of reelin positive cells in the entorhinal cortex, I ran a two-way ANOVA with

sex and treatment as the independent variables (Appendix C). All correlations were calculated using Pearson's correlation coefficient, with a simple linear regression line overlayed. To compare the novel object exploration DR against chance (DR=0) in the OBiPT, a one-sample t-test was used (Appendix C). Two-way ANOVAs were also used for both FST immobility and spleen WP area, with sex and condition as the independent variables for both (Appendix C). All data are reported as mean ± standard error of the mean (SEM). Significance in figures is denoted as follows: * = $p \leq 0.05$; ** = $p \leq 0.01$; and *** = $p \leq 0.001$.

3: Results

3.1: Reelin$^+$ cells in layer II of the EC

Nissl staining (Appendix B) visualises the six layers of the EC. Comparing this with the immunohistochemical staining of reelin$^+$ cells, their placement in layer II of the EC becomes visible (Figure 6). Reelin$^+$ cells can also be seen in high quantities in the hippocampus (Figure 6c), resulting from connections with the EC (Figure 2).

3.2: No change in density of reelin$^+$ cells in the EC following chronic stress

To examine the effect of chronic stress on reelin$^+$ cell density in the EC, and the effect of reelin injections on the reelin positive cell density after chronic stress, I compared the differences in reelin$^+$ cell density (Table 1) between sexes and between treatment groups (Figure 7). I found no significant effect of sex ($p = 0.07$), treatment group ($p = 0.15$), or interaction between effects ($p = 0.57$). Figure 8 shows examples of reelin$^+$ cells for each of the 6 groups.

3.3: No intact spatial memory observed through the OBiPT

To examine the effect of chronic stress on spatial memory, I compared the mean discrimination ratio for each of the six groups against chance: DR=0.0 (Figure 9). No group showed a DR value significantly different from 0 (all $p > 0.13$), and there was high variability.

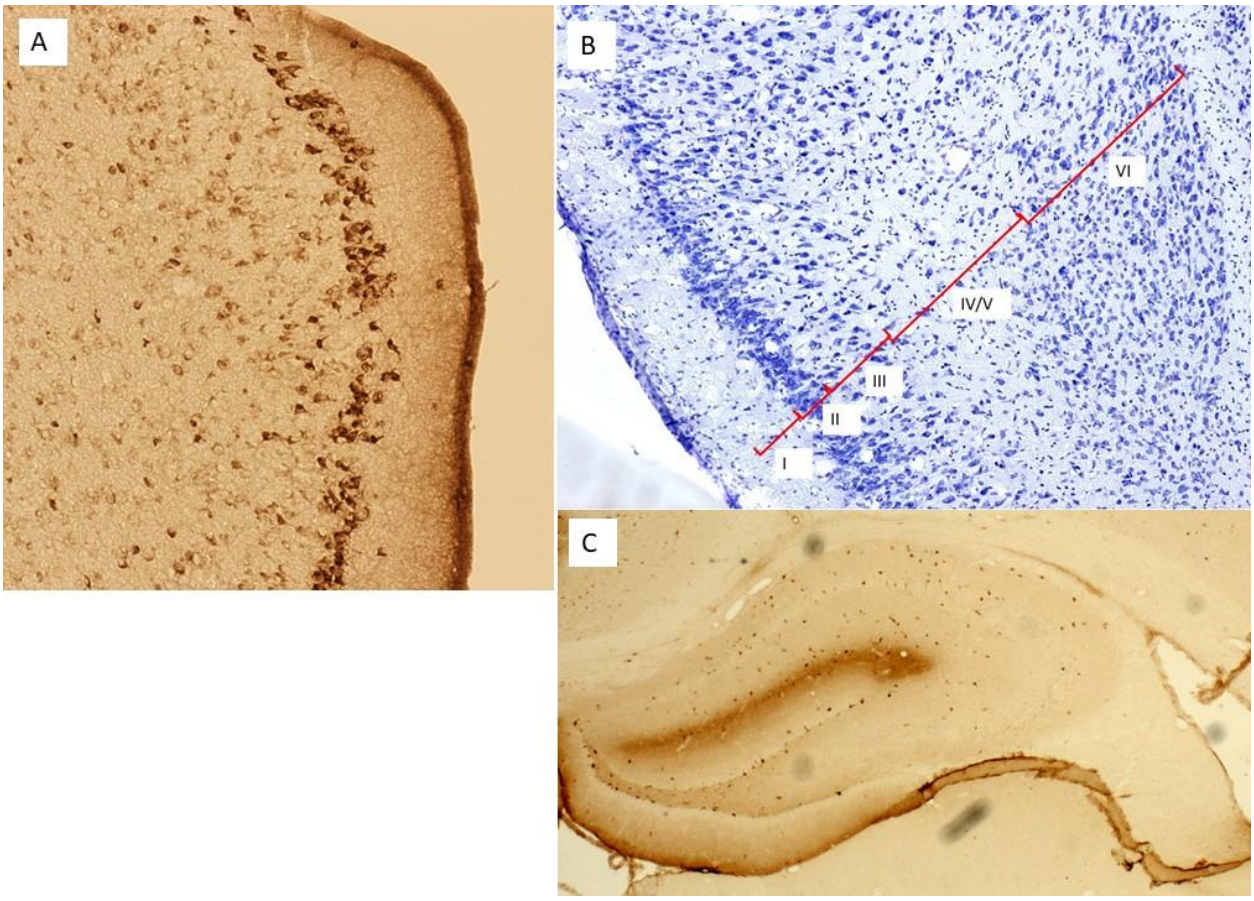

Figure 6. 20µm slices from a rat brain. A. Immunohistochemical staining showing the reelin^{+} cells in layer II of the LEC. B. Nissl staining showing the 6 layers of the LEC. C. Immunohistochemical staining showing the reelin^{+} cells in the hippocampus.

Table 1 Mean and SE of reelin^{+} cell densities in the EC for each sex and condition. No significant differences between groups. VV: vehicle/vehicle, CV: CORT/vehicle, CR: CORT/reelin.

	VV (cells/mm^2)	CV (cells/mm^2)	CR (cells/mm^2)
Males	1202.96 ± 234.995	1241.48 ± 123.40	1368.89 ± 136.13
Females	1462.22 ± 91.74	1296.30 ± 122.98	1739.26 ± 147.74

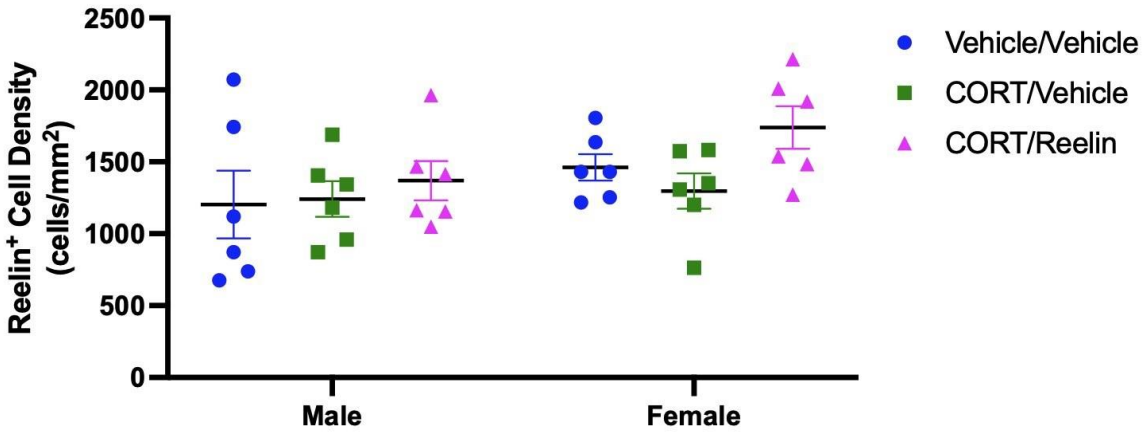

Figure 7. Counts of reelin^{+} cells in the EC expressed as density. No significant differences between any groups. Three conditions across two sexes (n=6 per sex per group). Mean, error bars: SEM.

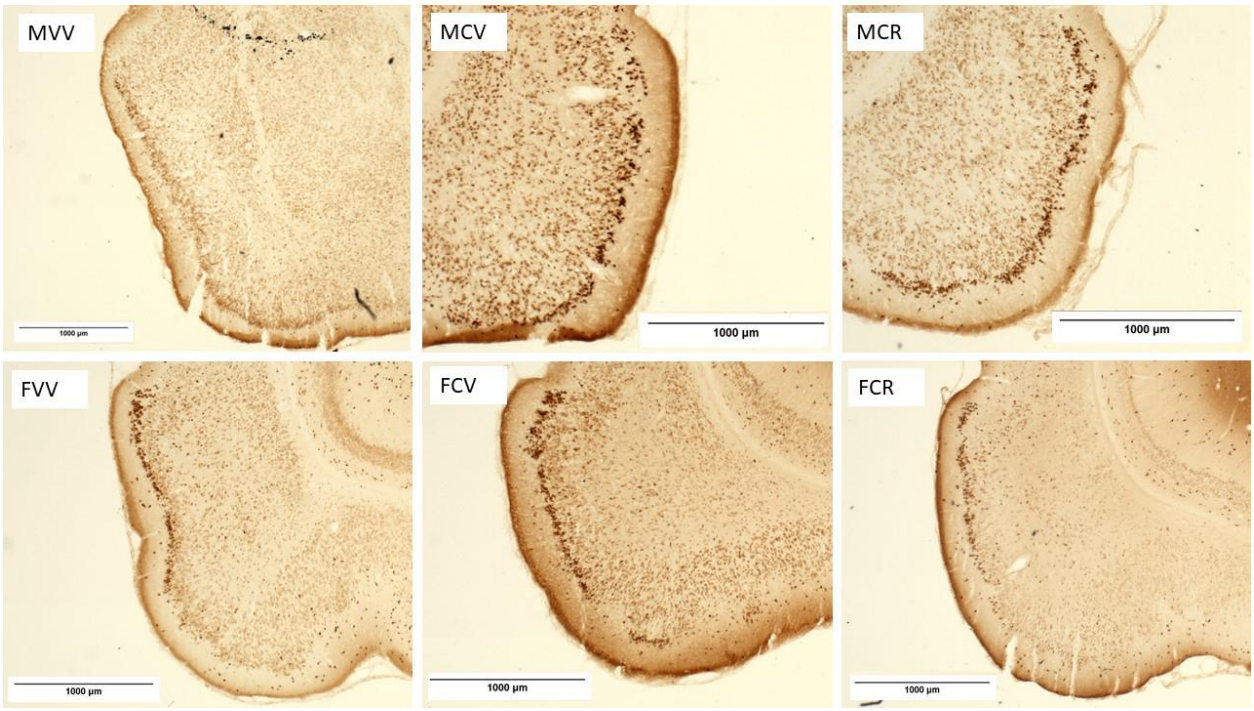

Figure 8. Examples of reelin^{+} cells in layer II of the EC for each of the 6 groups stained with immunohistochemistry. VV: vehicle/vehicle, CV: CORT/vehicle, CR: CORT/reelin; M: male; F: female. All scale bars = 1mm.

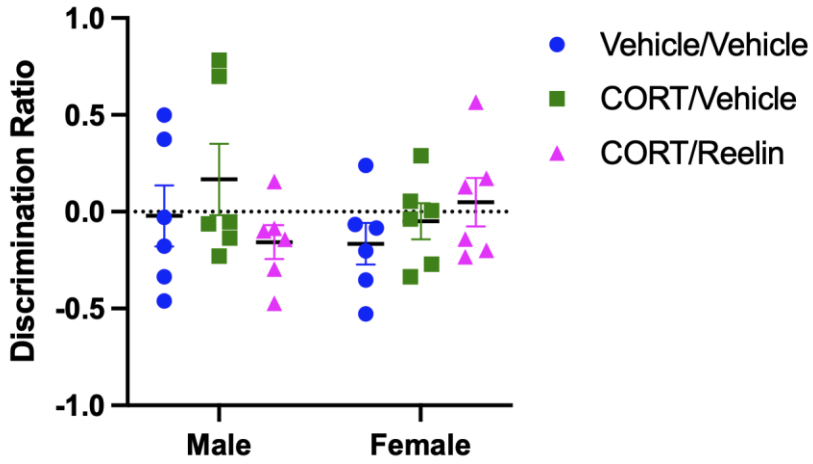

Figure 9. Discrimination ratio for each sex and condition. 0.0 is considered chance (dotted line). No condition scored significantly different than chance, with high variability. Mean, error bars: SEM.

3.4: Positive correlation between reelin$^+$ cell densities in the EC and spatial memory

I examined the relationship between the density of reelin$^+$ cells in the EC and the spatial memory DR scores observed. Significant correlations were found for MVV ($p = 0.021$; $r = 0.880$) and MCR ($p = 0.049$; $r = 0.813$) groups, with lower reelin$^+$ cell density related to a lower DR (Figure 10). No significant correlations were present for MCV ($p = 0.12$) or any of the female groups (all $p > 0.097$).

3.5: Increased FST immobility time following chronic stress

To see the effect of chronic stress on depressive-like behaviour, I compared the FST immobility time between sexes and the three treatment groups (Figure 11). Condition ($p < 0.0001$) showed a significant effect, whereas sex ($p = 0.119$) and interaction between sex and condition ($p = 0.311$) did not. *Post hoc* testing showed a significant increase in MCV immobility (231.55 ± 18.49s) compared to MVV (136.77s ± 15.16s) ($t(30) = 3.48$, $p = 0.014$). MCR (179.8s ± 6.55s) was not significantly different relative to either MCV ($p = 0.061$) or MVV ($p > 0.999$). As with males, the females showed no difference between FVV (110.12s ± 11.59s) and FCR (125.35s ± 7.09s) ($p > 0.999$), however FCV (236.99s ± 37.7s) was significantly increased relative to both

FVV ($t(30) = 4.66$, $p < 0.0001$) and FCR ($t(30) = 4.1$, $p = 0.003$). When repeating the comparisons to consider body mass, there was no change in differences between treatment groups. There was a significant difference between sexes, with FCV having significantly higher immobility time than MCV. The lack of significant changes between groups when accounting for body mass resulted in the decision to only use immobility time for the remainder of tests (see Appendix A: Reive et al., 2024 for detailed comparisons).

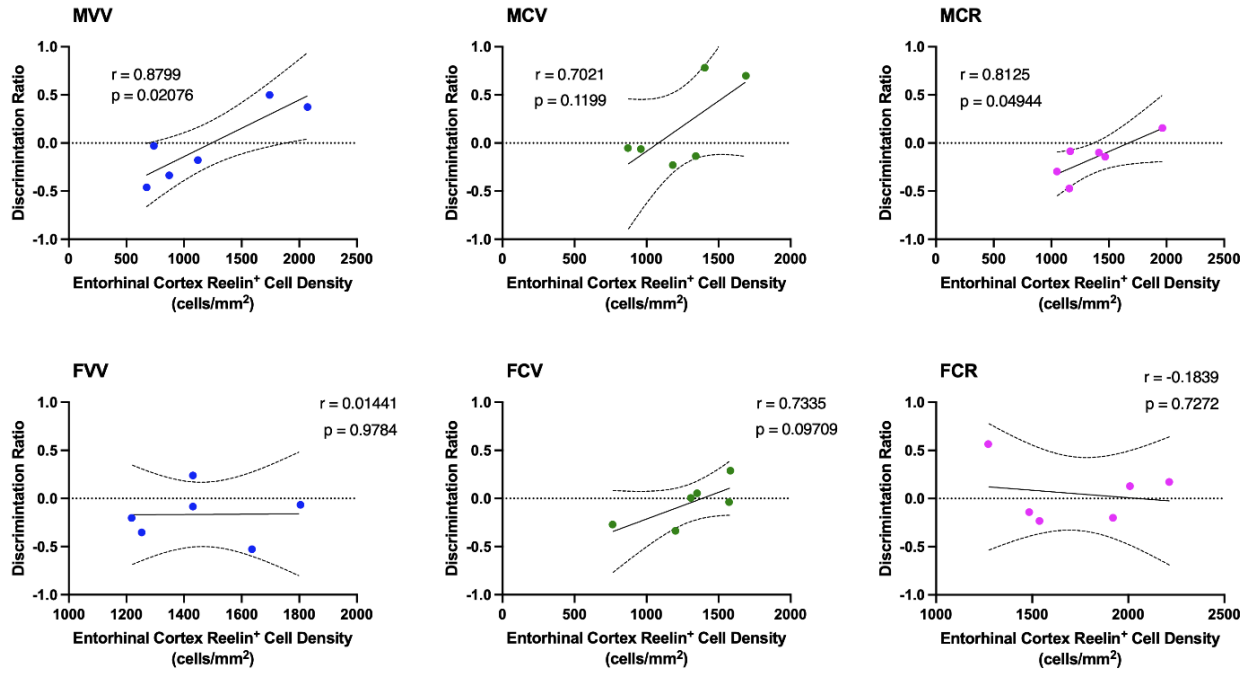

Figure 10. Correlations between reelin$^+$ cell density in the EC and DR scored from the OBiPT, separated by sex and condition (n=6). Significant positive correlations are present in MVV and MCR conditions, but not for any others. Solid line: simple linear regression; dotted lines: 95% CI. VV: vehicle/vehicle, CV: CORT/vehicle, CR: CORT/reelin; M: male; F: female.

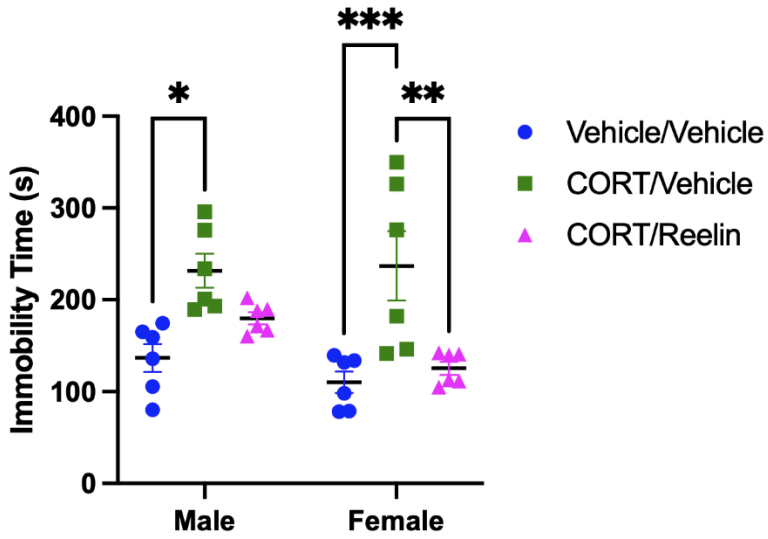

Figure 11. Immobility time as measured with the FST. Both male and female CV conditions show significantly increased immobility time relative to control (VV). Female CR condition also shows significantly reduced immobility time relative to CV. The male CR group is reduced, but not significantly so, and no differences present between sexes. Mean, error bars: SEM; * = $p \leq 0.05$; ** = $p \leq 0.01$; and *** = $p \leq 0.001$.

3.6: No correlation between FST immobility time and reelin$^+$ cell density in the EC

I examined the relationship between the immobility time and the density of reelin$^+$ cells in the EC following chronic stress, to see if the lack of reelin alterations was related to the depressive like behaviour observed (Figure 12). There were no significant correlations in any of the six groups (all $p > 0.3$).

3.7: Decreased spleen WP area following chronic stress

To compare the spleen WP cross sectional surface area between groups, I ran a two-way ANOVA. This revealed a significant effect of condition ($p = 0.002$), but not of sex ($p = 0.313$) or interaction between effects ($p = 0.26$) (Figure 13). *Post hoc* tests showed MCV (26.855% ± 0.87) had a significant reduction in WP area relative to both MVV (32.1% ± 1.36) ($t(30) = 3.684$, $p = 0.0027$) and MCR (30.56% ± 0.6596) ($t(30) = 2.599$, $p = 0.043$). MVV and MCR were not significantly different ($p=0.86$). There were no significant differences present between the female

treatment groups (all $p > 0.26$). Figure 14 demonstrates the WP area of the different conditions. See Reive et al. (2024) for further comparisons with body mass.

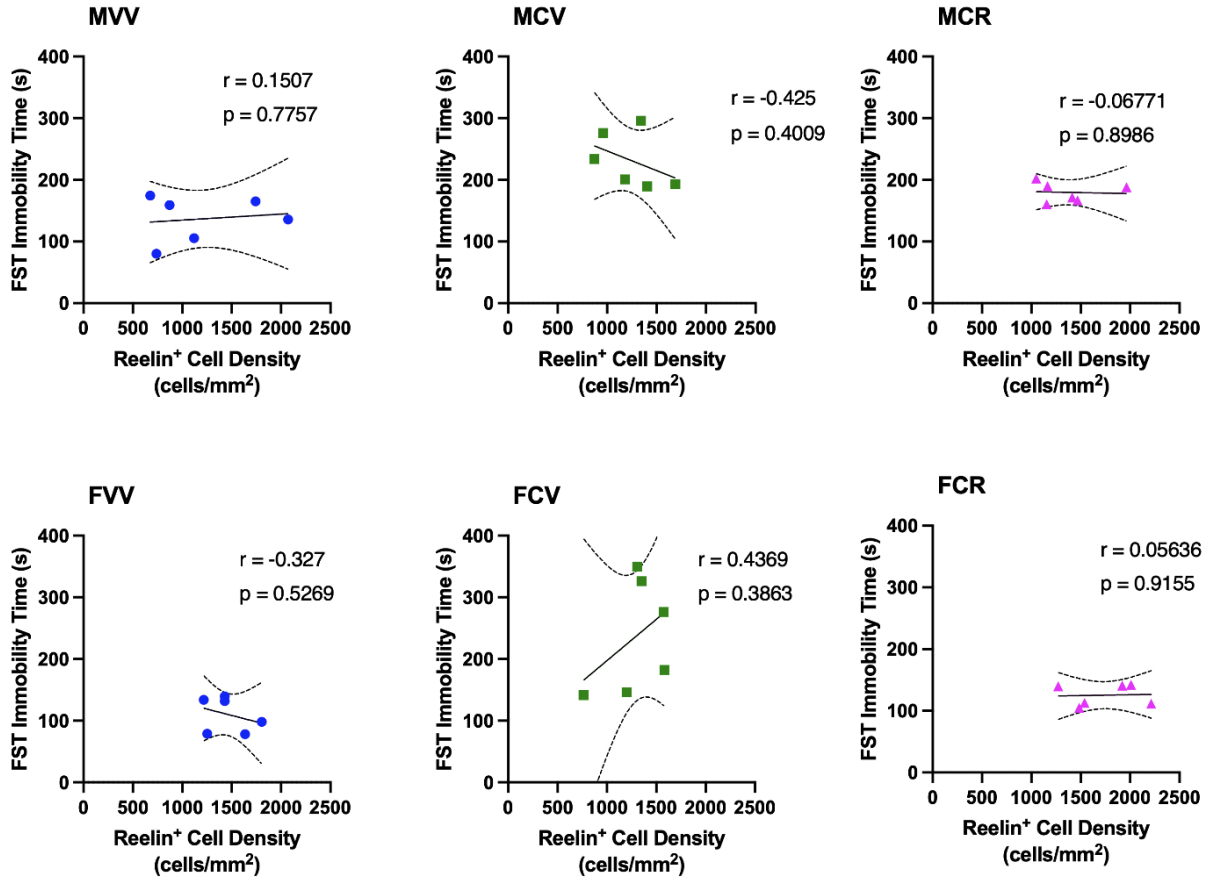

Figure 12. Correlations between reelin$^+$ cell density in the EC and immobility time in the FST, separated by sex and condition (n=6). No significant correlations found. Solid line: simple linear regression; dotted lines: 95% CI. VV: vehicle/vehicle, CV: CORT/vehicle, CR: CORT/reelin; M: male; F: female.

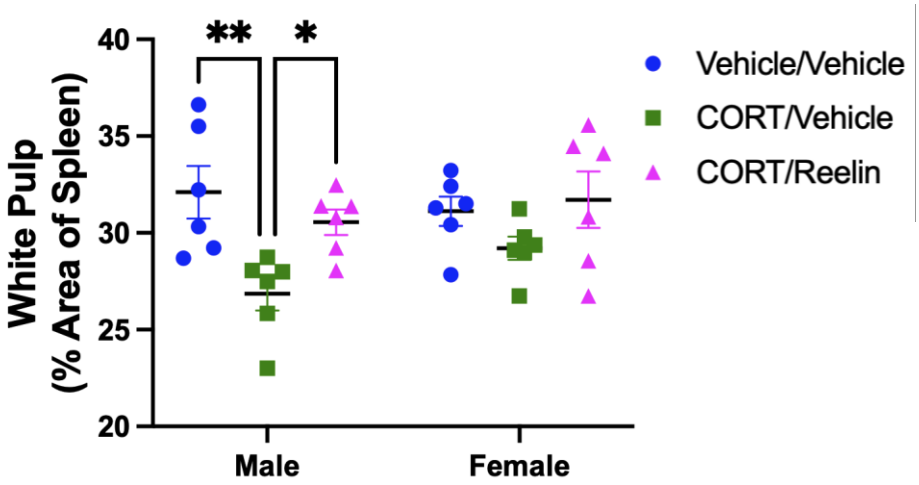

Figure 13. Spleen WP area for each condition and sex. Male CV group showed a significant reduction in WP area compared to both control and reelin group. No other significant differences present between conditions or sexes. Mean, error bars SEM; * = $p \leq 0.05$; ** = $p \leq 0.01$; and *** = $p \leq 0.001$.

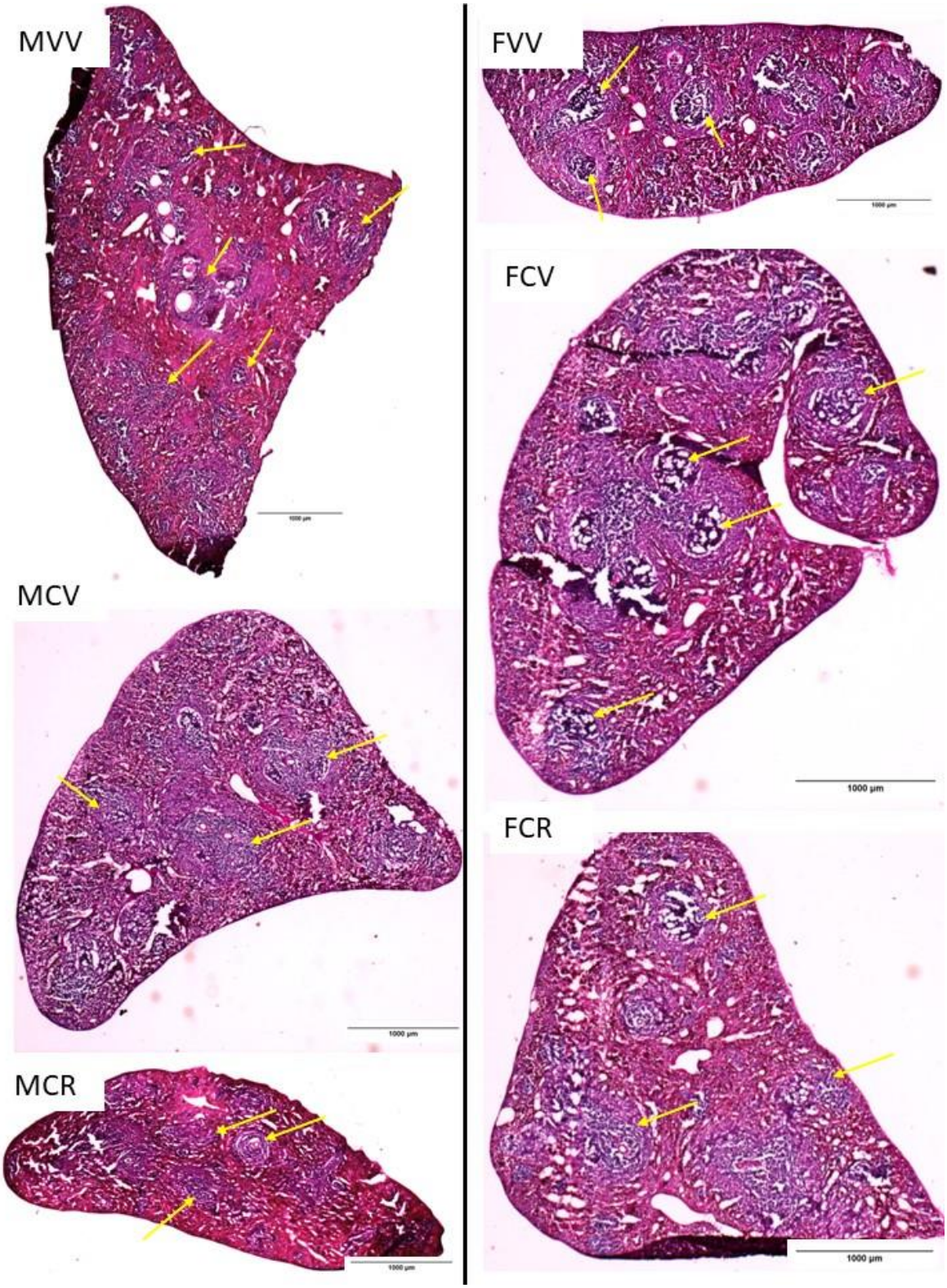

Figure 14. Examples of spleen cross-sections for each sex and condition, stained with HE. Purple regions are white pulp, marked by yellow arrows. VV: vehicle/vehicle, CV: CORT/vehicle, CR: CORT/reelin. All scale bars = 1mm.

3.8: Negative correlation between reelin$^+$ cell density in the EC and spleen WP area in females following chronic stress

I examined the relationship between the density of reelin$^+$ cells in the EC and the percentage of WP area in the spleen. The correlation between reelin positive cell density and WP area for the FCV group was significant, and negative ($p=0.035$, $r = -0.843$) (Figure 15). No other correlations for males or females showed significance (all $p > 0.182$).

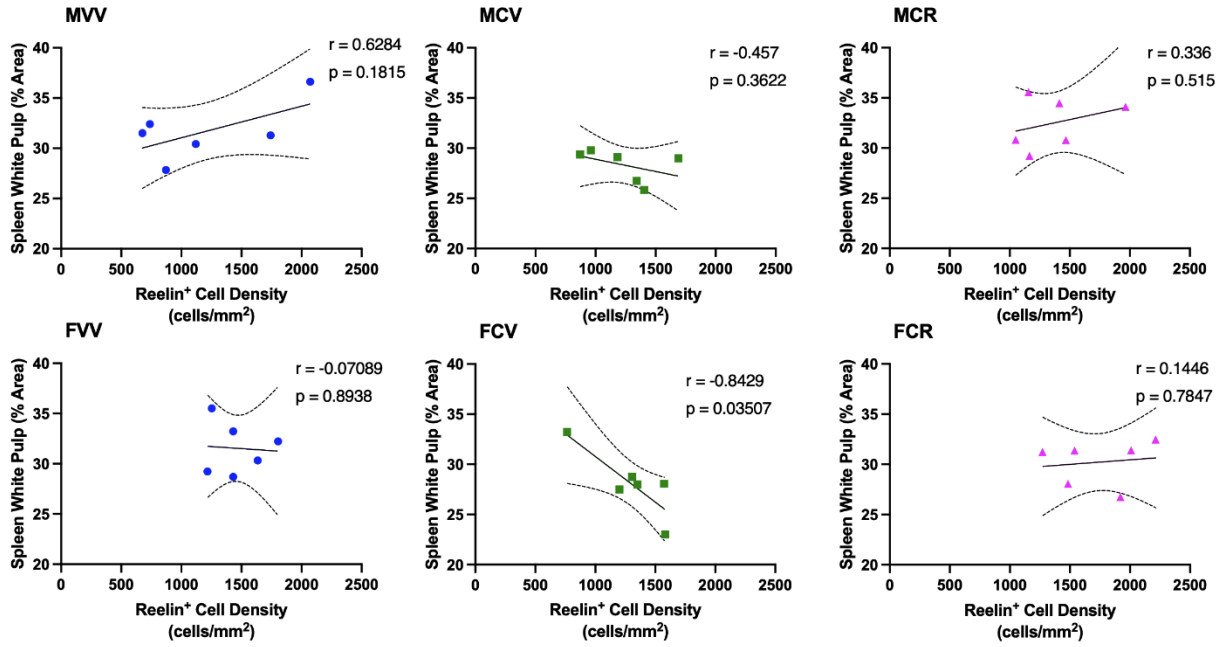

Figure 15. Correlations between spleen WP area and reelin$^+$ cell density in the EC. Significant negative correlations are present in the female CV group, but no others. Solid line: simple linear regression; dotted lines: 95% CI. VV: vehicle/vehicle, CV: CORT/vehicle, CR: CORT/reelin; M: male; F: female.

4: Discussion

4.1: Reelin in the EC

The results of this study showed that reelin$^+$ cell density was not depleted in the EC following chronic stress. Chronic stress reduces reelin in other brain regions following chronic stress, such as the dentate gyrus (Reive et al., 2024) and the hypothalamic paraventricular nucleus (Sánchez-Lafuente et al., 2022a). Additionally, treatment with intravenous reelin did not significantly affect reelin levels in the EC. Cell counts were marginally elevated in the CR condition compared to CV for both sexes, however these differences were not significant, due in part to the large variation. Reelin does show anti-depressant like properties following chronic stress through recovery of reelin$^+$ cell levels in the dentate gyrus (Allen et al., 2022; Reive et al., 2024). These suggest that chronic stress from CORT injections does not affect the EC in the same way that the hypothalamus and hippocampus are affected. Umegaki et al. (2003) proposed the EC to be more involved in processive stress responses (e.g., restraint stress) compared to systemic stress (e.g., hypoglycemia). This could be indicative of why no significant alterations were present, and using a model of processive stress such as restraint could yield different results. However, the reelin levels in the dentate gyrus do show changes following chronic stress (see Appendix A: Reive et al., 2024), and knowing that EC and hippocampus are highly connected, it could be worthwhile to perform further evaluation; for example looking at mRNA levels, or protein levels using a western blot.

4.2: The effect of chronic stress on cognition

The OBiPT showed no signs of intact spatial memory for any condition, including the control groups. This could be due to the parameters of the test relying on novelty preference instead of strictly spatial awareness, and that anhedonia is a symptom of depression, thus the differences

in time spent in each area could have been due to disinterest in the novel object locations. Another reason could be because all rats, including the control group, were under substantial social isolation stress. Rats are social creatures and are normally housed in pairs or groups. The stress induced from the CORT injections often results in increased aggression, so they were housed alone for the entirety of the 100-day study. Chronic social isolation, even much shorter durations, has negative effects on the behavioural and endocrinological responses of rats, and can reduce spatial memory (Krupina et al., 2020; Wang et al., 2019; Weiss et al., 2004).

While there were no signs of intact spatial memory, there was some correlation between the reelin$^+$ cells in the EC and cognition. Interestingly, this significant positive correlation was only seen in males, was disrupted by chronic stress, and was recovered by a single injection with reelin. This mimics the pattern of impairment from chronic stress and reelin-influenced recovery previously seen in reelin$^+$ cell levels, despair behaviour, and anxiety-like behaviour (Allen et al., 2022; Brymer et al., 2020; Reive et al., 2024). Research suggests that males and females tend to show similar spatial memory capabilities, with female skills being affected by sex hormone levels (Cost et al., 2012; Healy et al., 1999). The results from this study show females with a correlation pattern almost completely opposite that of the males. There are small sample sizes of only 6 rats per group and no significance is present, but the female CV group is the closest to having a significant correlation, with the VV and CR groups less so. This difference could stem from sex differences in spatial memory, sex differences in the effect of stressors or depression, or from something else entirely.

4.3: The effect of chronic stress on emotion

The FST was used to quantify depression-like behaviour. Measuring immobility time showed a clear pattern of increased immobility time for the stress (CV) group relative to both the

control (VV) and reelin (CR) conditions. This difference is much more pronounced in females compared to males, which is consistent with increased rate of depression in females (Mohammadi et al., 2023; Richards, 2011) and the findings of previous studies, such as Furtado et al. (2008) showing that while females have decreased EC volume in depression, males do not. The present recovery of mobility in the males receiving a reelin injection also lends additional support to the ability of reelin acting as an antidepressant, as previously demonstrated by this laboratory (Allen et al., 2022; Brymer et al., 2020; Johnston et al., 2023; Reive et al., 2024).

Knowing that depression can lead to decreased EC volume (Furtado et al., 2008) and decreased hippocampal reelin$^+$ cells (Fatemi et al., 2000), the correlations between depression-like behaviour (immobility time) and reelin$^+$ cell densities in the EC was examined, but nothing of significance was found. This could be due to the fact that the EC is more involved with cognitive roles such as of integration and processing, and less so emotional – for example a feeling of despair (Huang et al., 2023; Malone et al., 2024; Umegaki et al., 2003). Future research consisting of more cognition-based tests seems more likely to end up relating to the EC.

4.4: The effect of chronic stress on inflammation as evaluated by measurements of spleen white pulp atrophy

Measuring the area of WP in the spleens showed a pattern of reduction in WP area for the CV condition relative to the VV and CR conditions, however this was only significant for males. This supports previous research finding chronic stress can be involved in disruption of spleen WP (Avitsur et al., 2002; Hernandez et al., 2013; Vásquez et al., 2015; Zhang et al., 2021). Similarly, this further supports the ability of reelin administration to recover stress-induced changes, and is another example of sex-specific changes.

Disorders such as depression and AD show both inflammation and dysregulated levels of reelin (Caruncho et al., 2016; Fatemi et al., 2000; Herring et al., 2012; Lee & Giuliani, 2019; Liu et al., 2017). Correlating the reelin^{+} cell density in the EC with spleen WP area only showed a significant correlation with the FCV condition. Here, increased spleen WP area was correlated with decreased reelin^{+} cell density in the EC. This is another case of the CV condition showing different results than VV and CR, and another sex difference observed. Knowing that reelin plays a role in regulating immunity, and that immune responses often result in inflammation, it could then be speculated that this connection is what modulated the negative correlation in this situation (Alexander et al., 2023; Khialeeva & Carpenter, 2017). If the WP undergoes atrophy, disrupting the effectiveness of its ability to facilitate an immune response, then potentially the reelin upregulation is to support immunity (Alexander et al., 2023). This connection between WP atrophy and reelin would be valuable to explore further.

4.5: Sex differences – cognition, emotion, and inflammation

The results of this study showed a multitude of sex differences: in correlations between cognition and EC reelin levels, in correlations between spleen WP area and EC reelin levels, and in the stress-induced changes in immobility time and WP atrophy. This is likely due to natural sex differences for various reason. In humans, females are twice as likely to be diagnosed with depression (Mohammadi et al., 2023; Richards, 2011), however FST immobility times can be inconsistent in portraying this in rats (reviewed by Pitzer et al., 2022). The lack of recovery from reelin in females in both FST immobility time and spleen WP area could be due to the route of action of reelin as an antidepressant. Antidepressants, including ketamine, can vary in effectiveness between sexes (LeGates et al., 2019) and this could also be the case for reelin, given that reelin seems to act similarly to ketamine as an antidepressant (Johnston et al., 2023).

These results showed a correlation between reelin in the EC and cognition solely with males. Females have fluctuating sexual hormones, which are involved in many behavioural and biological responses, for example effectiveness of antidepressants (LeGates et al., 2019). Hormones can also affect cognitive ability in females (Laws et al., 2016). Females have been shown to have less cognitive abilities affected following stress, however they are more likely to get AD and show decreased cognition in AD compared to males (Laws et al., 2016; Luine et al., 2017). Females also show less endogenous reelin production, and AD often has dysregulated reelin (Chin et al., 2007; Sánchez-Lafuente et al., 2022b).

These results show sex differences in WP atrophy, with males showing greater stress-induced atrophy and recovery, and in the relation between WP area and reelin in the EC, with females showing a significant negative correlation. Sex differences in inflammation stemming from depression is not uncommon (Knight et al., 2020; Moieni et al., 2015). For example, Majd et al. (2018) found that heightened depressive symptoms corresponded with sex-specific inflammatory responses, with males showing increased inflammation and females decreased. Sexual hormones can be important in modulating immune responses, and can lead to sex differences (S. L. Klein & Flanagan, 2016)

4.6: Limitations and future directions

One limitation for this study is the lack of a baseline for counting area. Preliminary data collected demonstrated the necessity of manually placing boxes instead of performing unbiased stereography, as the latter resulted in a large variation in EC area counted. There was no baseline for the area counted per subject, thus arbitrary numbers were chosen. Additionally, there was a high level of background staining. This antibody is the same that has been used by this laboratory for the past decade, hence why no antibody controls were performed. This batch of antibody, unlike

previous ones received, resulted in much higher background staining levels while using the same protocol, thus adding an extra challenge when performing cell counts.

This study provides another example of strong sex differences in cognition and behaviour. It is worth considering this for a future direction, especially with the almost opposite correlations seen in spatial memory following chronic stress. To eliminate social isolation as a confounding factor, another group can be added that is housed in pairs or small groups instead of individually. Additionally, to increase the engagement of the EC in the stress response a paired stress model could be performed, where a cohort is exposed to both CORT and a restraint model simultaneously (Ngoupaye et al., 2018; Umegaki et al., 2003). Continuing the CORT paradigm for longer than 2.6 cycles could further demonstrate both the effects of CORT on depression like behaviour, as well as the efficacy of reelin as an antidepressant.

4.7: Relevance

Stress and depression are inherently linked, with stress being a risk factor for depression and similar biological mechanisms involved in both (Checkley, 1996). They both affect millions of people worldwide, with limited prescription drugs available. Learning more about the connections between molecular pathways (e.g., reelin), affected brain regions and their corresponding roles, and resulting behaviours in these disorders can guide future research towards preventions or treatments. This research highlights the connection between reelin, the EC, stress, and cognition. This could also have implications for future research surrounding AD, because this is a disease centered around a loss of cognitive ability, the EC is the first region showing neurodegeneration, and AD can show reelin dysregulation (Braak et al., 1993; Herring et al., 2012).

4.8: Conclusion

This study examined the effect of chronic stress on the density of reelin$^+$ cells in the EC with the cyclic CORT model of depression, and the therapeutic effect of a single final reelin injection. The study also evaluated the effect of chronic stress on cognition, emotion, and inflammation, and how each of these correlated with reelin$^+$ cell densities in the EC. Our hypotheses that chronic stress would alter reelin$^+$ cell density in the EC and be recovered with the reelin injection were not supported, there were no significant changes in density and thus no recovery could be seen. However, we did find significant correlations between reelin$^+$ cell density in the EC and cognition, which was disrupted with chronic stress and recovered with reelin injection in a sex-specific manner: we observed significant effects only in males. This was opposite for inflammation, where we saw a significant correlation solely for the female stress condition. No correlations between reelin$^+$ cell density in the EC and depression-like behaviour was found. All together, the results of this study support the idea of EC being important for spatial memory and cognition, the negative effects of chronic stress, and the sex-specific differences in depression, while showing that reelin is not altered in the EC with chronic stress. This is important in our understanding of the link between chronic stress and depression, the brain regions affected, and potentially the cognitive deterioration seen in AD.

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Appendix B: Nissl cresyl violet staining

Procedure used:

1. Distilled H_20 – brief dip
2. 0.1% cresyl violet (filtered) – 2 min
3. Distilled H20 – 30s
4. 70% EtOH (fresh) – 1 min
5. Differentiation: 95% EtOH and 1 drop acetic acid – 1.5 min
6. 95% EtOH – 1 min
7. 100% EtOH – 1 min
8. Xylene – 1 min
9. Xylene (refreshed) – 1 min
10. Xylene (refreshed) – 5 min
11. Coverslip with permount

Appendix C: Statistical analysis

1. ANOVA table: density of reelin+ cells in the EC

Table Analyzed	Density Analysis				
Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	**% of total variation**	**P value**	**P value summary**	**Significant?**	
Interaction	2.962	0.5703	ns	No	
SEX	9.027	0.0716	ns	No	
COndition	10.36	0.1527	ns	No	
ANOVA table	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**
Interaction	153718	2	76859	F (2, 30) = 0.5722	P=0.5703
SEX	468464	1	468464	F (1, 30) = 3.487	P=0.0716
COndition	537771	2	268886	F (2, 30) = 2.002	P=0.1527
Residual	4029812	30	134327		
Difference between row means					
Mean of Male	1271				
Mean of Female	1499				
Difference between means	-228.1				
SE of difference	122.2				
95% CI of difference	-477.7 to 21.35				

2. T test: OBiPT scores

One sample t and Wilcoxon test	A	B	C	D	E	F
	MVV	FVV	MCV	FCV	MCR	MCV
	Y	Y	Y	Y	Y	Y
1 Theoretical mean	0.5000	0.5000	0.5000	0.5000	0.5000	0.5000
2 Actual mean	0.4892	0.4172	0.5834	0.4754	0.4217	0.5246
3 Number of values	6	6	6	6	6	6
4						
5 **One sample t test**						
6 t, df	t=0.1376, df=5	t=1.538, df=5	t=0.9074, df=5	t=0.5291, df=5	t=1.805, df=5	t=0.3947, df=5
7 P value (two tailed)	0.8959	0.1846	0.4058	0.6194	0.1310	0.7094
8 P value summary	ns	ns	ns	ns	ns	ns
9 Significant (alpha=0.05)?	No	No	No	No	No	No
10						
11 **How big is the discrepancy?**						
12 Discrepancy	-0.01083	-0.08281	0.08337	-0.02461	-0.07834	0.02460
13 SD of discrepancy	0.1927	0.1318	0.2251	0.1139	0.1063	0.1527
14 SEM of discrepancy	0.07867	0.05383	0.09188	0.04651	0.04341	0.06233
15 95% confidence interval	-0.2131 to 0.191	-0.2212 to 0.05	-0.1528 to 0.319	-0.1442 to 0.094	-0.1899 to 0.03	-0.1356 to 0.184
16 R squared (partial eta squared)	0.003774	0.3213	0.1414	0.05302	0.3944	0.03021

3. ANOVA table: FST

	2way ANOVA ANOVA results					
1	Table Analyzed	FST - Immobility Time Total				
2						
3	**Two-way ANOVA**	Ordinary				
4	Alpha	0.05				
5						
6	**Source of Variation**	**% of total variation**	**P value**	**P value summary**	**Significant?**	
7	Interaction	3.433	0.3114	ns	No	
8	Row Factor	3.649	0.1188	ns	No	
9	Condition	50.48	<0.0001	****	Yes	
10						
11	**ANOVA table**	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**
12	Interaction	5388	2	2694	F (2, 30) = 1.213	P=0.3114
13	Row Factor	5726	1	5726	F (1, 30) = 2.579	P=0.1188
14	Condition	79223	2	39611	F (2, 30) = 17.84	P<0.0001
15	Residual	66609	30	2220		
16						
17	**Difference between row means**					
18	Mean of Male	182.7				
19	Mean of Female	157.5				
20	Difference between means	25.22				
21	SE of difference	15.71				
22	95% CI of difference	-6.853 to 57.30				

4. ANOVA table: WP area

1	Table Analyzed	White Pulp Both sexes				
2						
3	**Two-way ANOVA**	Ordinary				
4	Alpha	0.05				
5						
6	**Source of Variation**	**% of total variation**	**P value**	**P value summary**	**Significant?**	
7	Interaction	5.777	0.2602	ns	No	
8	Sex	2.163	0.3126	ns	No	
9	Condition	30.54	0.0024	**	Yes	
10						
11	**ANOVA table**	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**
12	Interaction	17.14	2	8.569	F (2, 30) = 1.409	P=0.2602
13	Sex	6.417	1	6.417	F (1, 30) = 1.055	P=0.3126
14	Condition	90.60	2	45.30	F (2, 30) = 7.447	P=0.0024
15	Residual	182.5	30	6.083		
16						
17	**Difference between row means**					
18	Mean of Male	29.84				
19	Mean of Female	30.68				
20	Difference between means	-0.8444				
21	SE of difference	0.8221				
22	95% CI of difference	-2.523 to 0.8346				