

Sildenafil, alone and in combination with imipramine or escitalopram, display antidepressant-like effects in an adrenocorticotrophic hormone-induced (ACTH) rodent model of treatment-resistant depression

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ABSTRACT

Background: Major depressive disorder (MDD) represents a challenge with high prevalence and limited effectiveness of existing treatments, particularly in cases of treatment-resistant depression (TRD). Innovative strategies and alternative drug targets are therefore necessary. Sildenafil, a selective phosphodiesterase type 5 (PDE5) inhibitor, is known to exert neuroplastic, anti-inflammatory, and antioxidant properties, and is a promising antidepressant drug candidate.

Aim: To investigate whether sildenafil monotherapy or in combination with a known antidepressant, can elicit antidepressant-like effects in an adrenocorticotrophic hormone (ACTH)-induced rodent model of TRD.

Methods: ACTH-naïve and ACTH-treated male Sprague-Dawley (SD) rats received various sub-acute drug treatments, followed by behavioural tests and biochemical analyses conversant with antidepressant actions.

Results: Sub-chronic ACTH treatment induced significant depressive-like behaviour in rats, evidenced by increased immobility during the forced swim test (FST). Sub-acute sildenafil (10 mg/kg) (SIL-10) (but not SIL-3), and combinations of imipramine (15 mg/kg) (IMI-15) and sildenafil (3 mg/kg) (SIL-3) or escitalopram (15 mg/kg) (ESC-15) and SIL-3, exhibited significant antidepressant-like effects. ACTH treatment significantly elevated hippocampal levels of brain-derived neurotrophic factor (BDNF), serotonin, norepinephrine, kynurenic acid (KYNUA), quinolinic acid (QUINA), and glutathione. The various mono- and combined treatments significantly reversed some of these changes, whereas IMI-15 + SIL-10 significantly increased glutathione disulfide levels. ESC-15 + SIL-3 significantly reduced plasma corticosterone levels.

Conclusion: This study suggests that sildenafil shows promise as a treatment for TRD, either as a stand-alone therapy or in combination with a traditional antidepressant. The neurobiological mechanism underlying the antidepressant-like effects of the different sildenafil mono- and combination therapies reflects a multimodal action and cannot be explained in full by changes in the individually measured biomarker levels.

1. Introduction

Major depressive disorder (MDD) is a serious mood disorder, characterized by persistent depressive symptoms, often accompanied by anhedonia and other distressing symptoms (American Psychiatric

Association, 2013; National Institute of Mental Health, 2023). Its impact on individuals and society is profound, given its chronic and recurrent nature (Reiersen et al., 2011; Ten Have et al., 2018; Verhoeven et al., 2018). It ranks as a leading contributor to global disability (Preoboth, 2000; Friedrich, 2017) and affects approximately 4.4% of the global

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population (World Health Organisation, 2017). Treatment-resistant depression (TRD) represents a particularly challenging and persistent form of MDD, characterized by severe, longer-lasting symptoms and a high recurrence rate (Rush et al., 2008; Cleveland, 2023), resulting in reduced quality of life, impaired social, cognitive, and occupational functioning, and an elevated risk of suicide (Hawton et al., 2013; Bergfeld et al., 2018; Lex et al., 2019; Gregory et al., 2020; Li, 2023).

Pharmacological treatment of MDD typically involves selective serotonin reuptake inhibitors (SSRIs) and other antidepressant classes (Malhi et al., 2013). For TRD, treatment strategies include optimization of pharmacotherapy, novel approaches such as ketamine or psilocybin, or augmentation with lithium or second-generation antipsychotics (Al-Harbi, 2012; Voineskos et al., 2020). However, limitations include side effects, delayed onset of action, and difficulties in managing cognitive deficits (Rosenzweig-Lipson et al., 2007; Carvalho et al., 2016). Notably, almost one-third of MDD patients do not achieve remission even after multiple trials of traditional antidepressants, who are classified as treatment-resistant (Rush et al., 2006; Zorumski et al., 2015; Carvalho et al., 2016). This underscores the need for novel and more effective treatment options.

Existing antidepressants primarily target central monoaminergic neurotransmission, particularly serotonergic, noradrenergic, and dopaminergic systems (Malhi et al., 2013; Willner et al., 2013; Boku et al., 2018). However, the pathophysiology of MDD may involve complex and diverse mechanisms (Duarte-Silva et al., 2020), with inflammation and oxidative stress playing a central role in MDD, and contributing to pathological features of the condition such as neurodegeneration, cell death, reduced neurogenesis, and autoimmune responses (Bakunina et al., 2015; Brand et al., 2015). An imbalance between reactive oxygen and nitrogen species and antioxidants, such as glutathione, can lead to oxidative stress and inflammation (Bakunina et al., 2015). Additionally, glutamate-related pathology is intimately linked in TRD, e.g., its response to ketamine (Schwartz et al., 2016; Phillips et al., 2019). Indeed, glutamate's association with the kynurenine pathway and subsequent effects on excitotoxicity via quinolinic acid-kynurenine imbalance, are causally linked to inflammation and changes in monoaminergic signalling, particularly serotonin (Niciu et al., 2014; Moller et al., 2015).

Glutamatergic signalling is linked to the nitric oxide (NO)-cyclic guanosine monophosphate (cGMP) cascade in depression, especially relapse and treatment refractoriness (Harvey, 1996; Harvey et al., 2002, 2006b). NO-cGMP in turn emerges as a critical player in neuro-modulation, neurotransmission, and redox-inflammatory signalling (Schulman, 1997; Calabrese et al., 2004; Straub et al., 2007; Banach et al., 2011). The cGMP/protein kinase G (cGMP/PKG) pathway, under the regulation of phosphodiesterase type 5 (PDE5), plays a pivotal role in modulating cyclic adenosine monophosphate (cAMP)-response element binding protein/brain-derived neurotrophic factor (CREB/BDNF) signalling and neuroplasticity (Feil et al., 2005; Dhir and Kulkarni, 2007). The antidepressant-like properties of selective PDE5 inhibitors, such as sildenafil, have been demonstrated in our laboratories (Brink et al., 2008; Liebenberg et al., 2010a, 2010b), supported by other pre-clinical studies (Baek et al., 2011; Matsushita et al., 2012; Tomaz et al., 2014; Wang et al., 2014; Socala et al., 2016). In this study, we explored the dose-dependent antidepressant-like effects of sildenafil, either alone or in combination with a known monoaminergic antidepressant, in an adrenocorticotrophic hormone-induced (ACTH) rodent model of TRD (Kitamura et al., 2002, 2008; Walker et al., 2013; Pereira et al., 2019). We hypothesised that (1) low-dose sildenafil and high-dose sildenafil in combination with imipramine would induce antidepressant-like effects and (2) low-dose sildenafil would augment the antidepressant-like effects of traditional monoaminergic antidepressants, i.e., imipramine and escitalopram, in the ACTH model of TRD.

2. Materials and methods

2.1. Animals

This study received approval from the Animal Care, Health, and Safety Research Ethics Committee (NWU-AnimCareREC; South African National Health Research Ethics Council reg. no.: AREC-130913-015) of the Faculty of Health Sciences, North-West University, South Africa (ethics approval no.: NWU-00598-19-A5).

Male SD rats ($n = 108$) were bred, supplied, and housed at the Animal Centre (Vivarium) of the Pre-Clinical Drug Development Platform (PCDDP) of the South African Department of Science and Innovation (DSI) and North-West University (NWU), South Africa (South African Veterinary Council (SAVC) registered (reg. no.: FR15/13458) and Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited (international file #1717)). All rats were group-housed (2–3 rats/cage) in $395 \times 346 \times 213$ mm ($w \times d \times h$) individually ventilated polysulphone cages under conditions of constant temperature (22 ± 1 °C), humidity ($55 \pm 10\%$) and positive air pressure. The Vivarium air was exchanged 16 to 18 times (fresh uncirculated air) per hour, and the air quality was controlled with high-efficiency particulate air (HEPA) filters. A 12-h light/dark cycle (lights on between 06:00 and 18:00) was maintained. Cages were cleaned, and bedding (chipped corncob) was replaced weekly. The rats had access to a polyvinyl chloride (PVC) tunnel shelter and nesting material (paper towel) as environmental enrichment. Food (standard rat chow) and tap water were provided *ad libitum*. The rats and all experimental facilities were protected from outside noise.

2.2. Drug treatments

During the sub-chronic treatment period, ACTH-(1–24) (ChinaPeptides Co., Ltd, Shanghai, China) was administered to the rats for 14 consecutive days at a fixed dosage of 0.1 mg/rat/day (Kitamura et al., 2008; Walker et al., 2013; Pereira et al., 2019). Then, rats received sub-acute administration of the following drugs alone and in different combinations: (1) imipramine hydrochloride (Sigma-Aldrich®, Schnelldorf, Germany) at 15 mg/kg (IMI-15) (Sales et al., 2011; Pereira et al., 2019), (2) escitalopram oxalate (Sigma-Aldrich®, Schnelldorf, Germany) at 15 mg/kg (ESC-15) (Mokoena et al., 2015) and (3) sildenafil citrate (Sigma-Aldrich®, Schnelldorf, Germany) at 3 mg/kg (SIL-3) or 10 mg/kg (SIL-10) (Liebenberg et al., 2010a). All rats were weighed daily during the sub-acute treatment period, with a dose adjustment calculation performed immediately before administration to enable accurate dosing in each rat. ACTH and drugs were all dissolved in phosphate-buffered saline (PBS) and subsequently administered via subcutaneous injection. The ACTH crystals were dissolved in a constant volume of PBS (0.1 ml), whereas the drug powders were dissolved in a maximum of 0.25 ml of PBS, using the smallest volume possible. Control rats received only vehicle control (VEH), i.e., PBS, as did the ACTH-naïve control rats.

Previous studies from our laboratories informed the dosages selected for sildenafil and specific classes of traditional antidepressants chosen to constitute part of the combination therapies with sildenafil in this study. These studies found that the antidepressant-like properties of sildenafil display strict dose dependency, with only higher doses (≥ 10 mg/kg) exhibiting an interaction with the cholinergic system in rodents (Brink et al., 2008; Liebenberg et al., 2010a, 2010b). Hence, the antidepressants imipramine (having anticholinergic properties) and escitalopram (not having anticholinergic properties) were used to investigate whether low- and/or high-dosage sildenafil can augment the antidepressant-like effects of an antidepressant with anticholinergic properties and one without these properties, thereby overcoming antidepressant treatment resistance in ACTH-treated SD rats. See Brink et al. (2008) for a detailed discussion of the role that the central cholinergic system plays in the antidepressant-like effects of sildenafil (Brink et al., 2008).

2.3. Experimental design

All rat pups were weaned on postnatal day 21 (PND21) and randomly allocated to home cages (3 rats/cage). Rat pups were randomly divided into an ACTH-naïve and ACTH-treated group comprising 12 and 96 rats, respectively. Afterward, they were randomly divided into sub-acute treatment groups comprising 12 rats each, using the same procedure described above. All the rat pups, therefore, had an equal chance to be allocated to a specific home cage, the ACTH-naïve or ACTH-treated group, and any of the various sub-acute treatment groups, preventing possible cage effects. Importantly, the ACTH-naïve group comprised a single sub-acute treatment group, namely a VEH (negative control) [C1] group. However, the ACTH-treated group consisted of 8 different sub-acute treatment groups, including a VEH (negative control) [C2], IMI-15 (positive control) [D1], ESC-15 (positive control) [D2], SIL-3 [D3], SIL-10 [D4], IMI-15 + SIL-3 [D5], IMI-15 + SIL-10 [D6] and ESC-15 + SIL-3 [D7] group. Fig. 1 below illustrates the study layout.

Starting on PND50, rats in the ACTH-naïve group daily received VEH (negative control) until PND63, whereas rats in the ACTH-treated group received ACTH. Sub-acute treatments followed immediately before the forced swim test (FST), as described below (Porsolt et al., 1977; Porsolt, 1979).

- **Dose 1:** VEH and drugs corresponding to the various treatment groups were administered between 20:00 and 20:30 on PND63 (24 h before the forced swim test (FST) performed on the following day),
- **Dose 2:** VEH and drugs corresponding to the various treatment groups were administered for a second time between 15:00 and 15:30 on PND64 (5 h before the FST performed on the same day), and
- **Dose 3:** VEH and drugs corresponding to the various treatment groups were administered for a third and final time between 19:00 and 19:30 on PND64 (1 h before the FST performed on the same day).

All the rats were habituated to (placed in) an open field test (OFT) arena for 10 min under red light (80 lux) on the evening of PND63,

whereas the OFT test procedure was performed 24 h later, on the evening of PND64. Similarly, a 15-min pre-conditioning swim session was carried out for all the rats under red light (80 lux) on PND63 (directly after the OFT habituation session), followed 24 h later by the FST on PND64 (directly after the OFT test procedure). The OFT test procedure is usually performed in conjunction with the FST to eliminate possible drug-induced effects on the general locomotor activity of rats, viz., any changes in immobility observed during the FST can be ascribed to drug-induced changes in psychomotor and not general locomotor activity (Saayman et al., 2021). All rats were humanely euthanised on PND65 (at least 12 h following completion of the FST performed on the previous evening to eliminate the immediate effects that swimming stress may have on biochemical marker levels), whereafter plasma and brain (hippocampus) samples were collected and stored at -80°C for subsequent biochemical analyses. Finally, corticosterone levels were analysed in the plasma, whereas BDNF, serotonin, norepinephrine, KYNUA, QUINA, glutathione, and glutathione disulfide were analysed in the hippocampus.

2.4. Rodent model of antidepressant treatment resistance

ACTH (0.1 mg/rat) was administered to rats daily between 17:00 and 19:00 through a single subcutaneous injection for 14 days, as described in previous studies (Kitamura et al., 2008; Walker et al., 2013; Pereira et al., 2019). This model is based on evidence suggesting that sub-chronic ACTH treatment negates the antidepressant-like action of monoaminergic antidepressants in novelty suppressed feeding (Antunes et al., 2015) and forced swim tests (FST) (Kitamura et al., 2002, 2008; Walker et al., 2013; Pereira et al., 2019). Therefore, the ACTH model of TRD possesses sound predictive validity, while its etiological and construct validity is founded on the underlying role of hypercortisolaemia in the pathophysiology of the disorder (Hornig-Rohan et al., 1996; Markopoulou et al., 2009; Markopoulou, 2013; Caraci et al., 2018).

2.5. Statistical analyses

Power analyses were performed to determine the minimum number

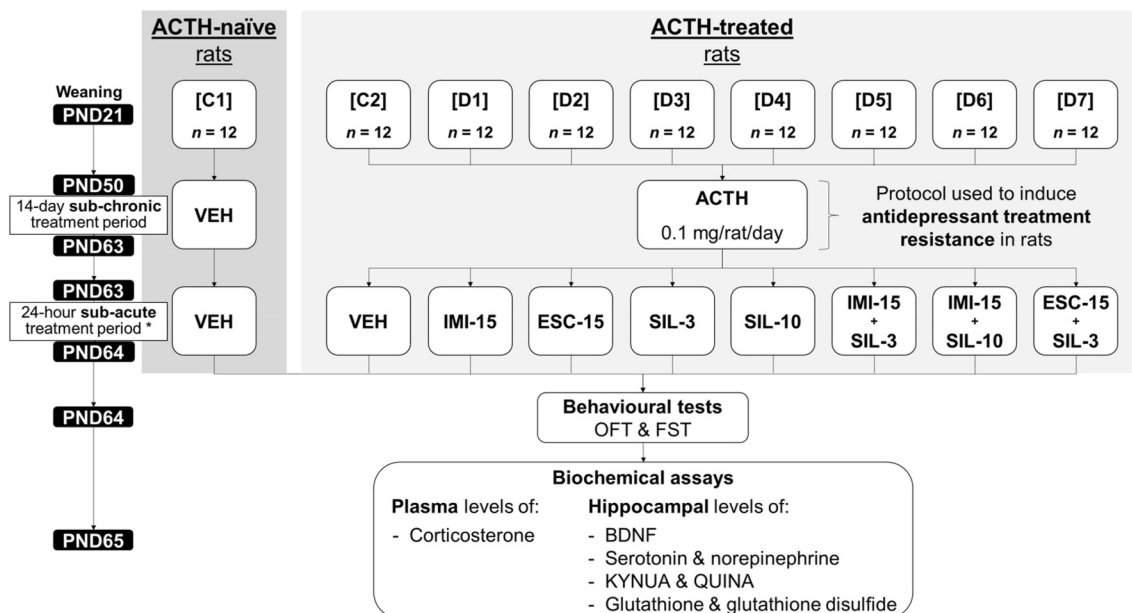


Fig. 1. A schematic illustration of the study layout. ACTH: adrenocorticotrophic hormone. BDNF: brain-derived neurotrophic factor. ESC-15: escitalopram oxalate (15 mg/kg). FST: forced swim test. IMI-15: imipramine hydrochloride (15 mg/kg). KYNUA: kynurenic acid. n : number of rats per treatment group. OFT: open field test. PND: postnatal day. QUINA: quinolinic acid. SIL-3: sildenafil citrate (3 mg/kg). SIL-10: sildenafil citrate (10 mg/kg). VEH: vehicle control. * A total of 3 VEH or drug doses were administered during this period (24, 5, and 1 h before the FST was performed).

of rats needed per treatment group to ensure statistically meaningful results. In cases where a one-way analysis of variance (ANOVA) was performed, power calculations were conducted considering the omnibus test (F statistic), and the standardised effect size was defined as medium to large ($F = 0.25$ and $F = 0.40$, respectively). Power calculations were conducted in all cases considering a type-I error rate of 5% ($\alpha = 0.05$). The threshold for type II error was 20% ($1 - \beta \geq 0.8$). Power calculations were conducted using G*power version 3.1.9.2.

GraphPad Prism® version 8 for Windows (GraphPad Prism® software, Version 8.0, San Diego, California, United States of America) was used for all statistical analyses and graphical representations. Grubbs' test was performed on all data sets to screen for outliers (with $\alpha = 0.05$ accepted as significant). The Shapiro-Wilk's and Levene's tests were conducted to test for normality of distribution and homogeneity of variances (with $p < 0.05$ accepted as a violation of both assumptions), respectively. An unpaired Student's t -test (data distributed normally) or Mann-Whitney U test (data not distributed normally) was used to determine the effects of sub-chronic ACTH treatment in rats (by comparing the ACTH-treated to the ACTH-naïve control group). Furthermore, to assess the impact of different sub-acute treatments in ACTH-treated rats, an Ordinary one-way ANOVA (multiple comparisons for data distributed normally) or Kruskal-Wallis one-way ANOVA (numerous comparisons for data not distributed normally) was performed (by comparing the various sub-acute treatment groups to each other). Ordinary one-way ANOVAs were followed by a Dunnett's post-hoc test for multiple comparisons. In contrast, Kruskal-Wallis one-way ANOVAs were followed by a Dunn's test (comparing the various sub-acute drug treatment groups to the VEH treatment group in ACTH-treated rats). Data are presented as the mean $\pm$ standard error of the mean (SEM). Statistical significance was set at $p \leq 0.05$ for all comparisons.

Statistical analyses were followed by effect magnitude calculations, where appropriate. Effect magnitudes indicate strong trends and minimise Type I (false positive) or Type II (false negative) errors, specifically in instances where the assumption of homogeneity of variances is violated. The unbiased Cohen's d -value (d) was used to calculate the effect magnitude of interactions and intergroup differences (with a 95% confidence interval (CI) of the effect magnitude). The d was calculated to establish the practical (clinical) significance of effect magnitude. Only medium ($d \geq 0.6$) and large ($d \geq 0.8$) effect sizes were considered significant. Effect magnitude indicators were calculated in Exploratory Software for CIs (Cohen, 1988).

2.6. Behavioural tests

Prior studies in our laboratory have shown that preceding behavioural tests do not influence the outcome of subsequent consecutive tests when behavioural tests are performed from least to most stressful (Mokoena et al., 2015). Thus, the OFT was performed (50 min after the third and final dose of VEH or drug treatment) immediately before the FST. As rats are nocturnal animals, behavioural tests were performed during the dark cycle (between 18:00 and 06:00). More specifically, to accommodate for the initial foraging and activity of rats, behavioural tests only commenced 1 h after the start of the dark cycle (at 19:00).

2.6.1. Open field test (OFT)

The OFT test procedure is performed to evaluate the general locomotor activity of rodents and, hence, the ability to move around and negotiate their surroundings (Schoeman et al., 2017). Since more anxious rodents will tend to remain stationary in a corner or next to a wall of the OFT arena (Ramos et al., 1997; Prut and Belzung, 2003; Hiroi and Neumaier, 2006), increased anxiety-like behaviour in the OFT (neophobia, e.g., freezing) (Misslin and Cigrang, 1986) may influence general locomotor activity as assessed in the OFT. As a result, the habituation of rats in the OFT arena (24 h before performing the OFT test procedure) was performed to negate this possibility.

The OFT apparatus consisted of four $100 \times 100 \times 45$ cm ($w \times d \times h$) square arenas inscribed on the floor, with opaque black walls. A video camera situated directly above each OFT arena recorded locomotor behaviour. The test was performed on PND64 under red light (80 lux), as previously described (Schoeman et al., 2017; Saayman et al., 2021). In short, each rat was placed in the centre of an OFT arena and allowed 5 min for exploration. Following each test session, the rats were returned to their home cages, and whereafter the OFT arenas were wiped with a 10% ethanol solution to eliminate any olfactory cues during subsequent test sessions. The video recordings were scored using EthoVision XT 14 software (Noldus Information Technology BV, Wageningen, Netherlands), with the total distance moved (cm) used to measure general locomotor activity.

2.6.2. Forced swim test (FST)

The FST is routinely performed to evaluate antidepressant-like activity for a broad spectrum of antidepressants (Borsini and Meli, 1988). The test is based on deficits in escape-directed behaviour when rats are subjected to an inescapable water cylinder and the reversal thereof by an antidepressant. Adopting an immobile posture, i.e., immobility, in the FST has been associated with a failure of perseverance in escape-directed behaviour (behavioural despair) and reflects depressive-like behaviour (Porsolt et al., 1977; Porsolt, 1979). A pre-conditioning swim on day 1 is required to sensitize the animals, which subsequently present with behavioural despair when reintroduced to the FST on day 2 of the test (Pereira et al., 2019; Saayman et al., 2021).

The FST apparatus consisted of four 20×100 cm ($d \times h$) cylindrical tanks spaced next to each other, each filled to a depth of 40 cm with ambient water (maintained at 25 ± 1 °C), and a video camera situated directly in front of the four FST cylinders. The FST was performed on PND64 under red light (80 lux), as previously described (Schoeman et al., 2017; Pereira et al., 2019). In short, each rat was placed into a water-filled FST cylinder and allowed to swim for 7 min while being video recorded, following which the rats were removed from the cylinders, dried with a towel, and returned to their home cages. The water in the FST cylinders was replaced with clean water following every FST trial to avoid any influence of odour trails on subsequent FST trials (Abel and Bilitzke, 1990). The video recordings were scored using EthoVision XT 14 software (Noldus Information Technology BV, Wageningen, Netherlands), with total time spent immobile (sec) used to measure their depressive-like behaviour. However, the first and last minutes of the FST were not scored (leaving a total of 5 min that were scored) to maximise the accuracy of the results (Oberholzer et al., 2018). If necessary, the investigator was out of sight but close at hand to intervene should there be signs of untoward submersion.

2.7. Biochemical assays

Specific biomarkers, including plasma corticosterone and hippocampal BDNF, serotonin, norepinephrine, KYNUA, QUINA, glutathione, and glutathione disulfide levels, were analysed, as described in sections 2.7.1.1., 2.7.2.1. and 2.7.2.2.

2.7.1. Blood collection and processing

Following the euthanasia of rats by decapitation (without anaesthesia) on PND65, their trunk blood was collected in pre-chilled 4 ml BD Vacutainer® tubes containing 7.2 mg K₂-EDTA (dipotassium ethylenediaminetetraacetic acid). The blood-filled tubes were manually mixed before being placed on ice. The blood samples were subsequently centrifuged (at 14 000 rpm and 4 °C for 10 min), whereafter the plasma layer (top layer) was pipetted into 1.5 ml Eppendorf® microcentrifuge tubes and subsequently preserved at -80 °C until the plasma corticosterone assay (Möller et al., 2013).

2.7.1.1. Plasma corticosterone assay. Plasma samples were analysed for corticosterone using liquid chromatography-mass spectrometry (LC-MS) as described previously (Li et al., 2014; Yuan et al., 2015).

2.7.2. Brain tissue collection and preparation

Immediately after euthanasia, the whole brain of individual rats was extracted and placed in ice-cold PBS. The hippocampi were quickly

dissected on an ice-cooled dissection slab and placed into 1.5 ml Eppendorf® microcentrifuge tubes. The tubes were subsequently snap frozen in liquid nitrogen and preserved at -80°C until the hippocampal biomarker assays were performed (Harvey et al., 2006a; Brand and Harvey, 2017; Steyn et al., 2018).

2.7.2.1. Hippocampal brain-derived neurotrophic factor (BDNF) assay.

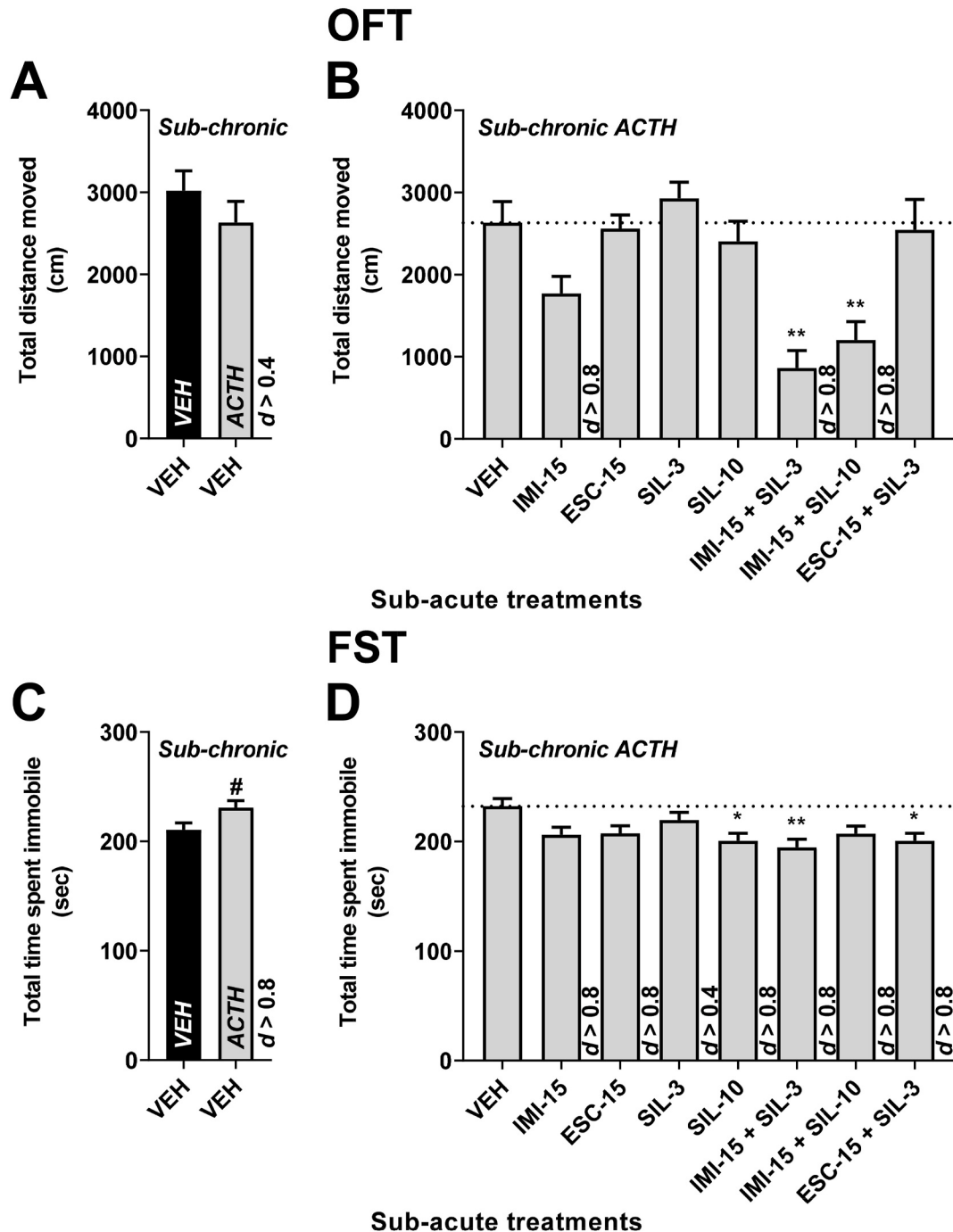


Fig. 2. A graphical representation of the OFT and FST data. A Distance moved by rats after receiving sub-chronic VEH or ACTH treatment, followed by sub-acute VEH treatment. B Distance moved by rats after receiving sub-chronic ACTH treatment, followed by sub-acute VEH or drug treatment. C Time spent immobile by rats after receiving sub-chronic VEH or ACTH treatment, followed by sub-acute VEH treatment. D Time spent immobile by rats after receiving sub-chronic ACTH treatment, followed by sub-acute VEH or drug treatment. All group sizes are equal ($n = 12$). Data points represent the mean $\pm$ SEM. Statistical analyses are reported in the text with d vs the VEH + VEH treatment group in A, $**p < 0.01$, and d vs the ACTH + VEH treatment group in B, # $p < 0.05$, and d vs the VEH + VEH treatment group in C, and $*p < 0.05$, $**p < 0.01$ and d vs the ACTH + VEH treatment group in D. ACTH: adrenocorticotropic hormone. d : unbiased Cohen's d -value. ESC-15: escitalopram oxalate (15 mg/kg). FST: forced swim test. IMI-15: imipramine hydrochloride (15 mg/kg). OFT: open field test. SIL-3: sildenafil citrate (3 mg/kg). SIL-10: sildenafil citrate (10 mg/kg). VEH: vehicle control.

Hippocampal BDNF levels were measured using rat BDNF enzyme-linked immunosorbent assay (ELISA) kits (catalogue no.: E-EL-R1235, Elabscience Biotechnology Inc.) according to the manufacturer instructions (Steyn et al., 2018; Saayman et al., 2021).

2.7.2.2. Hippocampal serotonin, norepinephrine, kynurenic acid (KYNUA), quinolinic acid (QUINA), glutathione, and glutathione disulfide assay. According to previous methods, hippocampal tissue samples were assayed for serotonin, norepinephrine, KYNUA, QUINA, glutathione, and glutathione disulfide using LC-MS (Moriarty et al., 2011; Squellierio et al., 2012; Fuertig et al., 2016; Wojnicz et al., 2016; Wang et al., 2019).

3. Results

As illustrated by graphs A, C, E, and G (see sections 3.1.1. and 3.2.1. to 3.2.5.), the ACTH-treated group is compared to the ACTH-naïve control group, with both these groups comprising SD rats that received 3 doses of VEH during the sub-acute treatment period of this study. Graphs B, D, F, and H respectively depict the same ACTH-treated groups as in graphs A, C, E, and G, which serve as the control groups for the ACTH plus various sub-acute drug treatment groups.

3.1. Behavioural analysis

Data were obtained from the behavioural tests performed on PND64 once all the VEH and drug treatments had been administered.

3.1.1. Distance moved and time spent immobile

Fig. 2 below depicts the total distance moved in the OFT and time spent immobile in the FST by SD rats after they received sub-chronic (over 14 days) VEH or ACTH treatment, followed by sub-acute (over 24 h) VEH or drug treatment. Since FST data are corrected for possible drug-induced changes in the general locomotor activity of rats using analyses of covariance (ANCOVAs) in this study, changes observed in the time spent immobile in the FST can be directly ascribed to drug-induced changes in psychomotor (and not general locomotor) activity.

An unpaired Student's t-test revealed no statistically significant difference between the ACTH + VEH (ACTH-treated) and the VEH + VEH (ACTH-naïve) control group regarding distance moved in the OFT ($p = 0.2874$, $d = 0.43$) (see Fig. 2A). A Kruskal-Wallis one-way ANOVA test for multiple comparisons indicated statistically significant differences between the various sub-acute treatment groups receiving ACTH regarding distance moved in the OFT ($p < 0.0001$). Dunn's post-hoc test for multiple comparisons showed in ACTH-treated rats that sub-acute IMI-15 + SIL-3 ($p = 0.0013$, $d = 2.09$) and IMI-15 + SIL-10 ($p = 0.0065$, $d = 1.65$) treatments reduce the distance moved in the OFT compared to VEH (see Fig. 2B).

An unpaired Student's t-test revealed a statistically significant difference between the ACTH + VEH and the VEH + VEH control group regarding time spent immobile in the FST. Sub-chronic ACTH treatment elevates the time spent immobile by SD rats in the FST compared to VEH ($p = 0.0322$, $d = 0.92$) (see Fig. 2C). An Ordinary one-way ANOVA test for multiple comparisons indicated, all in ACTH-treated rats, that statistically significant differences were evident between the various sub-acute treatment groups regarding time spent immobile in the FST ($F_{(7, 88)} = 2.983$, $p = 0.0074$). Dunn's post-hoc test for multiple comparisons showed, all in ACTH-treated rats, that sub-acute SIL-10 ($p = 0.0111$, $d = 1.30$), IMI-15 + SIL-3 ($p = 0.0016$, $d = 1.34$) and ESC-15 + SIL-3 ($p = 0.0108$, $d = 1.43$) treatments significantly reduce the time spent immobile in the FST compared to VEH-treated rats (see Fig. 2D).

3.2. Rat biochemical marker levels

Data were obtained from the various biochemical assays conducted

on PND65 once all the VEH and drug treatments had been administered and behavioural tests performed.

3.2.1. Plasma corticosterone levels

Fig. 3 below depicts the total plasma corticosterone levels of SD rats after receiving sub-chronic VEH or ACTH treatment, followed by sub-acute VEH or drug treatment.

An unpaired Student's t-test revealed no statistically significant difference in plasma corticosterone levels between the ACTH + VEH and the VEH + VEH control group ($p = 0.1655$) (see Fig. 3A). However, an effect magnitude calculation demonstrated a practical (clinical) significant difference between these groups regarding plasma corticosterone levels ($d = 0.61$; Fig. 3A). A Kruskal-Wallis one-way ANOVA test for multiple comparisons across ACTH-treated rats indicated statistically significant differences in plasma corticosterone levels between the various sub-acute treatment groups ($p < 0.0001$). Dunn's post-hoc test for multiple comparisons showed, all in ACTH-treated rats, that sub-acute ESC-15 + SIL-3 ($p = 0.0034$, $d = 1.28$) treatment significantly reduces plasma corticosterone levels compared to VEH-treated rats (see Fig. 3B).

3.2.2. Hippocampal brain-derived neurotrophic factor (BDNF) levels

Fig. 4 below depicts the total hippocampal BDNF levels of SD rats after receiving sub-chronic VEH or ACTH treatment, followed by sub-acute VEH or drug treatment.

An unpaired Student's t-test revealed a statistically significant difference between the ACTH + VEH and the VEH + VEH control group regarding hippocampal BDNF levels. Therefore, sub-chronic ACTH treatment significantly elevates hippocampal BDNF levels compared to VEH-treated SD rats ($p = 0.0074$, $d = 1.16$) (see Fig. 4A). An Ordinary one-way ANOVA test for multiple comparisons, all in ACTH-treated rats, indicated statistically significant differences between the various sub-acute treatment groups regarding hippocampal BDNF levels ($F_{(7, 87)} = 8.417$, $p < 0.0001$). Dunn's post-hoc test for multiple comparisons showed, all in ACTH-treated rats, that sub-acute IMI-15 ($p < 0.0001$, $d = 2.37$), ESC-15 ($p < 0.0001$, $d = 2.19$), SIL-10 ($p = 0.0336$, $d = 1.25$), IMI-15 + SIL-3 ($p < 0.0001$, $d = 1.51$), IMI-15 + SIL-10 ($p = 0.0002$, $d = 1.54$) and ESC-15 + SIL-3 ($p < 0.0001$, $d = 1.74$) treatments significantly reduce hippocampal BDNF levels compared to VEH-treated rats (see Fig. 4B).

3.2.3. Hippocampal serotonin and norepinephrine levels

Fig. 5 below depicts the total hippocampal serotonin and norepinephrine levels of SD rats after receiving sub-chronic VEH or ACTH treatment, followed by sub-acute VEH or drug treatment.

An unpaired Student's t-test revealed a statistically significant difference between the ACTH + VEH and the VEH + VEH control group regarding hippocampal serotonin levels. Therefore, sub-chronic ACTH treatment significantly elevates hippocampal serotonin levels compared to VEH-treated SD rats ($p = 0.0174$, $d = 1.01$) (see Fig. 5A). An Ordinary one-way ANOVA test for multiple comparisons, all in ACTH-treated rats, indicated statistically significant differences between the various sub-acute treatment groups regarding hippocampal serotonin levels ($F_{(7, 85)} = 3.521$, $p = 0.0023$). Dunn's post-hoc test for multiple comparisons showed, all in ACTH-treated rats, that sub-acute IMI-15 ($p = 0.0004$, $d = 1.40$), ESC-15 ($p = 0.0122$, $d = 1.30$), SIL-3 ($p = 0.0138$, $d = 1.25$), SIL-10 ($p = 0.0065$, $d = 1.22$) and ESC-15 + SIL-3 ($p = 0.0060$, $d = 1.26$) treatments significantly reduce hippocampal serotonin levels compared to VEH-treated rats (see Fig. 5B).

An unpaired Student's t-test revealed a statistically significant difference between the ACTH + VEH and the VEH + VEH control group regarding hippocampal norepinephrine levels. Therefore, sub-chronic ACTH treatment significantly elevates hippocampal norepinephrine levels compared to VEH-treated SD rats ($p = 0.0064$, $d = 1.22$) (see Fig. 5C). An Ordinary one-way ANOVA test for multiple comparisons indicated, all in ACTH-treated rats, statistically significant differences

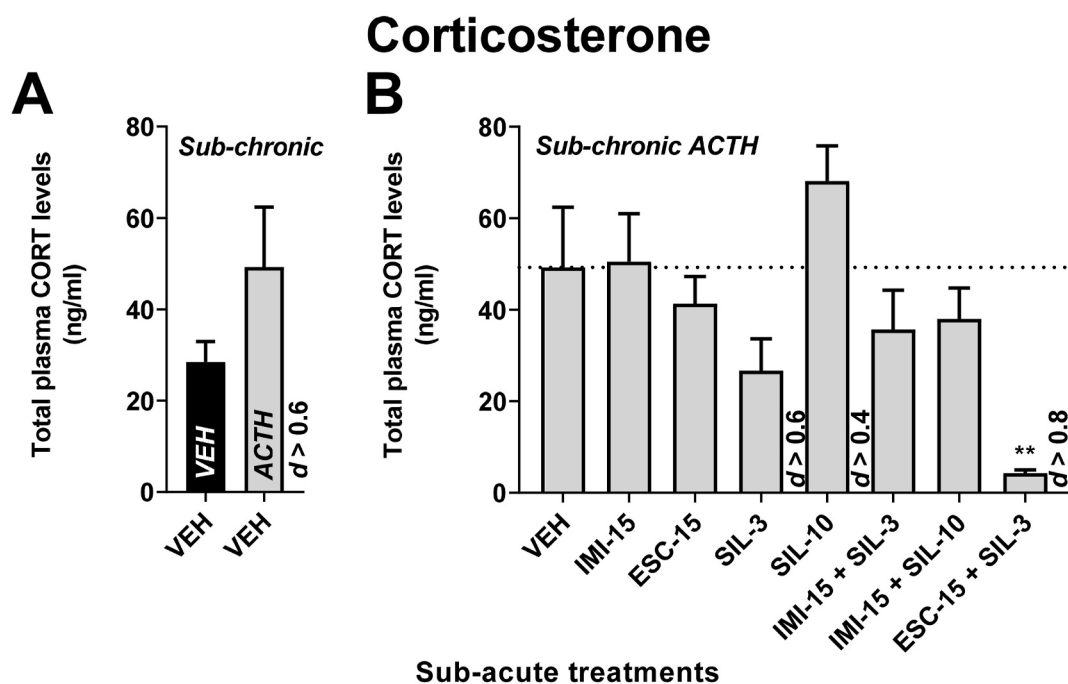


Fig. 3. A graphical representation of the plasma corticosterone assay data. **A** Corticosterone levels of rats after receiving sub-chronic VEH or ACTH treatment, followed by sub-acute VEH treatment. **B** Corticosterone levels of rats after receiving sub-chronic ACTH treatment, followed by sub-acute VEH or drug treatment. All group sizes are equal ($n = 12$). Data points represent the mean $\pm$ SEM. Statistical analyses are reported in the text with d vs the VEH + VEH treatment group in **A**, and $**p < 0.01$ and d vs the ACTH + VEH treatment group in **B**. ACTH: adrenocorticotrophic hormone. d : unbiased Cohen's d -value. ESC-15: escitalopram oxalate (15 mg/kg). IMI-15: imipramine hydrochloride (15 mg/kg). SIL-3: sildenafil citrate (3 mg/kg). SIL-10: sildenafil citrate (10 mg/kg). VEH: vehicle control.

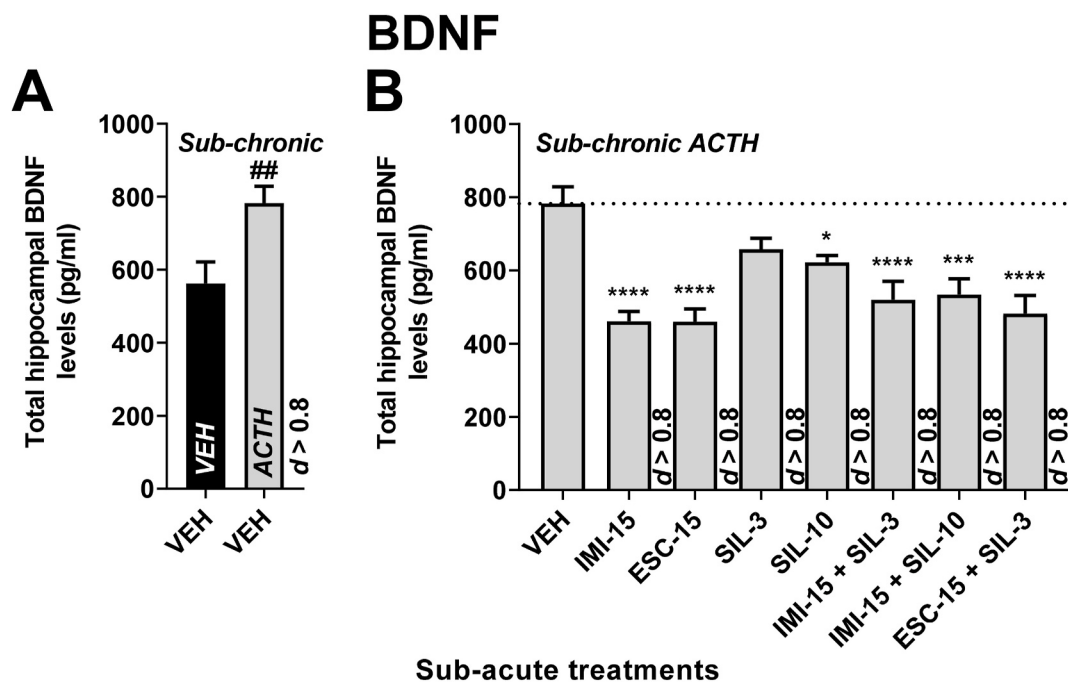


Fig. 4. A graphical representation of the hippocampal BDNF assay data. **A** BDNF levels of rats after receiving sub-chronic VEH or ACTH treatment, followed by sub-acute VEH treatment. **B** BDNF levels of rats after receiving sub-chronic ACTH treatment, followed by sub-acute VEH or drug treatment. All group sizes are equal ($n = 12$). Data points represent the mean $\pm$ SEM. Statistical analyses are reported in the text with $##p < 0.01$ and d vs the VEH + VEH treatment group in **A**, and $*p < 0.05$, $***p < 0.001$, $****p < 0.0001$ and d vs the ACTH + VEH treatment group in **B**. ACTH: adrenocorticotrophic hormone. BDNF: brain-derived neurotrophic factor. d : unbiased Cohen's d -value. ESC-15: escitalopram oxalate (15 mg/kg). IMI-15: imipramine hydrochloride (15 mg/kg). SIL-3: sildenafil citrate (3 mg/kg). SIL-10: sildenafil citrate (10 mg/kg). VEH: vehicle control.

between the various sub-acute treatment groups regarding hippocampal norepinephrine levels ($F_{(7, 87)} = 9.317$, $p < 0.0001$). Dunnett's post-hoc test for multiple comparisons showed in ACTH-treated rats that sub-

acute SIL-3 ($p = 0.0134$, $d = 1.01$) and SIL-10 ($p = 0.0417$, $d = 0.89$) treatments significantly reduced hippocampal norepinephrine levels compared to VEH-treated rats (see Fig. 5D).

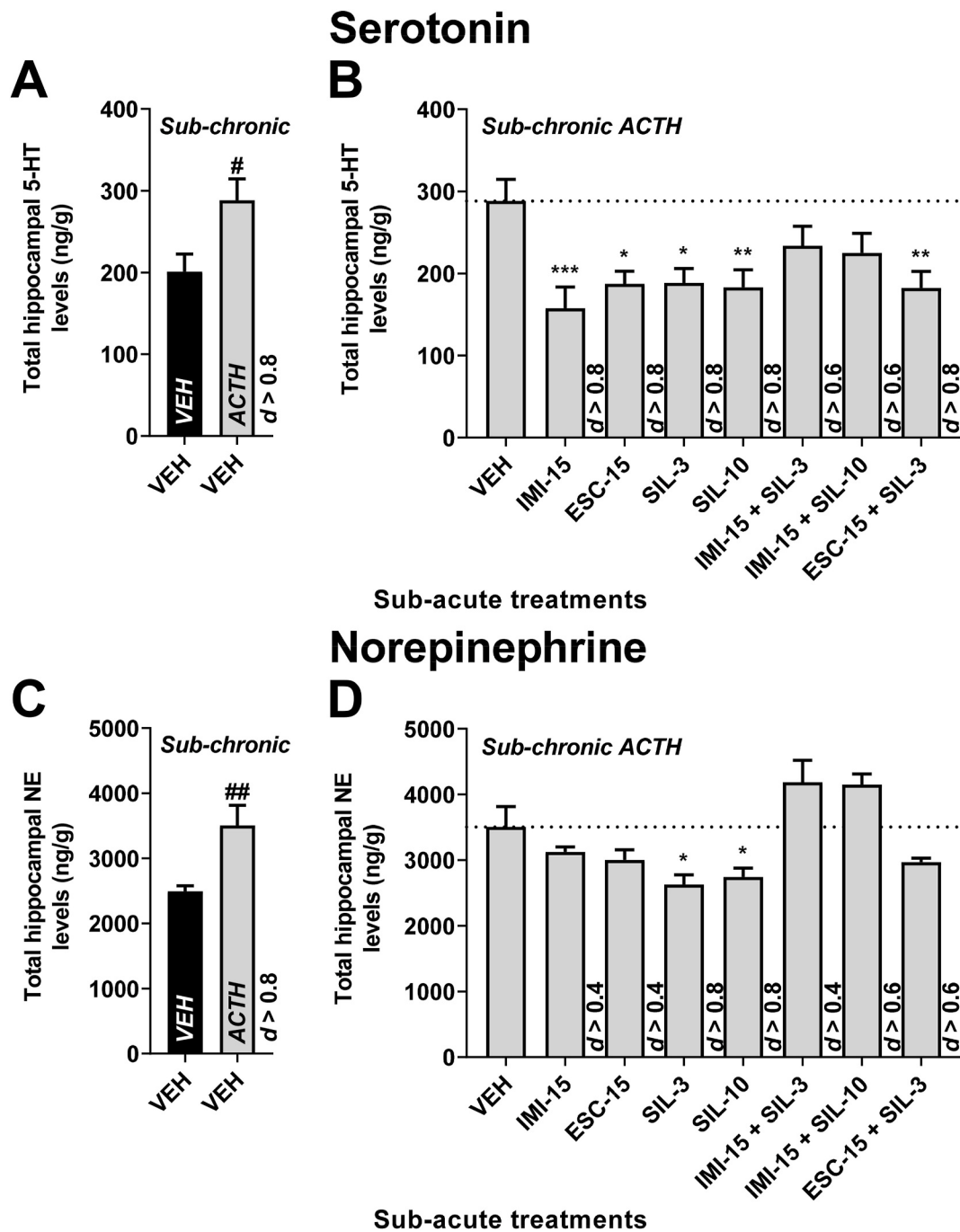


Fig. 5. A graphical representation of the hippocampal serotonin and norepinephrine assay data. **A** Serotonin levels of rats after receiving sub-chronic VEH or ACTH treatment, followed by sub-acute VEH treatment. **B** Serotonin levels of rats after receiving sub-chronic ACTH treatment, followed by sub-acute VEH or drug treatment. **C** Norepinephrine levels of rats after receiving sub-chronic VEH or ACTH treatment, followed by sub-acute VEH treatment. **D** Norepinephrine levels of rats after receiving sub-chronic ACTH treatment, followed by sub-acute VEH or drug treatment. All group sizes are equal ($n = 12$). Data points represent the mean $\pm$ SEM. Statistical analyses are reported in the text with $\#p < 0.05$ and d vs the VEH + VEH treatment group in **A**, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and d vs the ACTH + VEH treatment group in **B**, $##p < 0.01$ and d vs the VEH + VEH treatment group in **C**, and $*p < 0.05$ and d vs the ACTH + VEH treatment group in **D**. ACTH: adrenocorticotrophic hormone. d : unbiased Cohen's d -value. ESC-15: escitalopram oxalate (15 mg/kg). IMI-15: imipramine hydrochloride (15 mg/kg). SIL-3: sildenafil citrate (3 mg/kg). SIL-10: sildenafil citrate (10 mg/kg). VEH: vehicle control.

3.2.4. Hippocampal kynurenic acid (KYNUA) and quinolinic acid (QUINA) levels

Fig. 6 below depicts the total hippocampal KYNUA and QUINA levels of SD rats after receiving sub-chronic VEH or ACTH treatment, followed by sub-acute VEH or drug treatment.

An unpaired Student's t -test revealed a statistically significant difference between the ACTH + VEH and the VEH + VEH control group

regarding hippocampal KYNUA levels. Therefore, sub-chronic ACTH treatment elevates hippocampal KYNUA levels compared to VEH-treated SD rats ($p = 0.0041$, $d = 1.26$) (see Fig. 6A). An Ordinary one-way ANOVA test for multiple comparisons, all in ACTH-treated rats, indicated statistically significant differences between the various sub-acute treatment groups regarding hippocampal KYNUA levels ($F_{(7, 86)} = 11.84$, $p < 0.0001$). Dunnett's post-hoc test for multiple comparisons

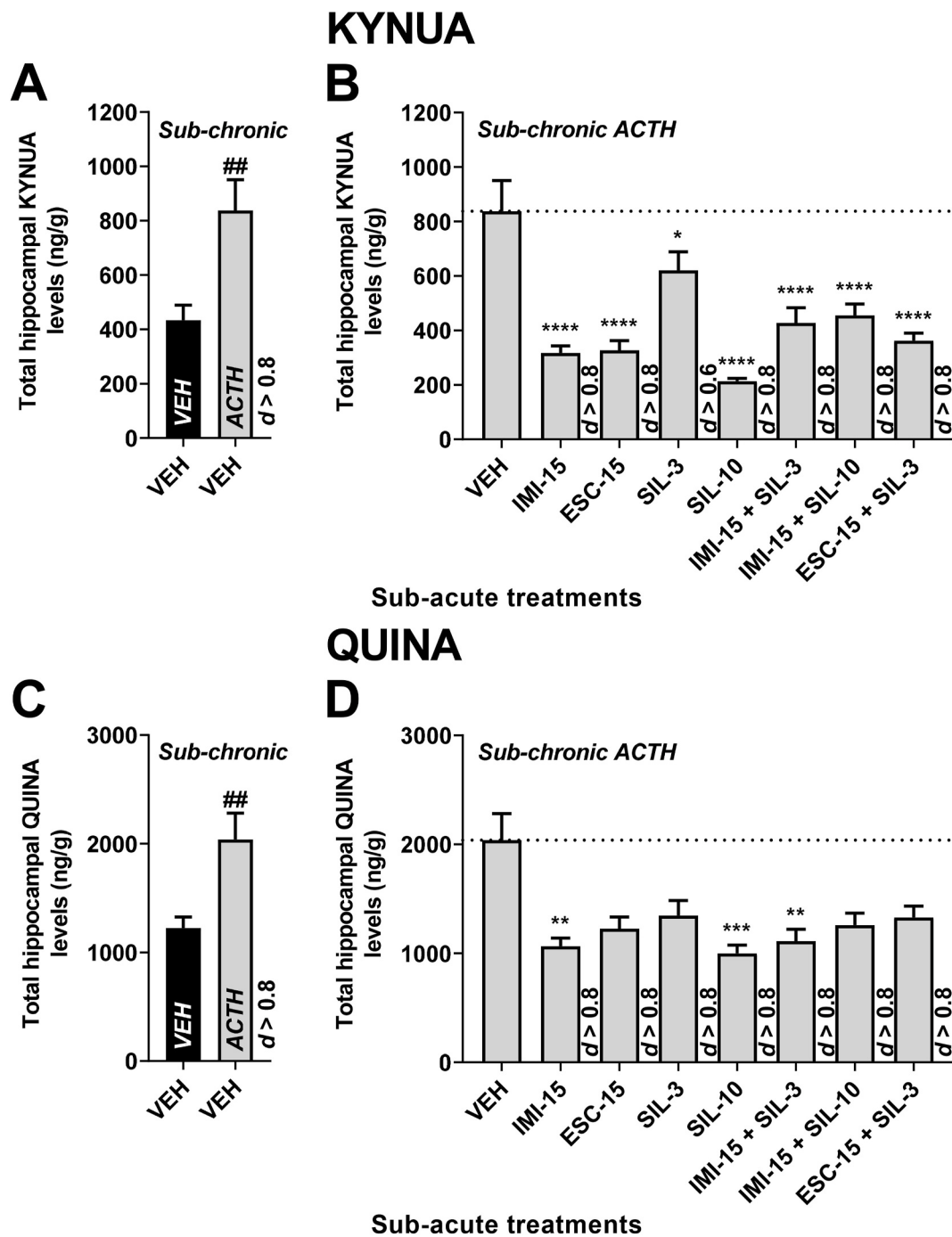


Fig. 6. A graphical representation of the hippocampal KYNUA and QUINA assay data. **A** KYNUA levels of rats after receiving sub-chronic VEH or ACTH treatment, followed by sub-acute VEH treatment. **B** KYNUA levels of rats after receiving sub-chronic ACTH treatment, followed by sub-acute VEH or drug treatment. **C** QUINA levels of rats after receiving sub-chronic VEH or ACTH treatment, followed by sub-acute VEH treatment. **D** QUINA levels of rats after receiving sub-chronic ACTH treatment, followed by sub-acute VEH or drug treatment. All group sizes are equal ($n = 12$). Data points represent the mean $\pm$ SEM. Statistical analyses are reported in the text with $##p < 0.01$ and d vs the VEH + VEH treatment group in **A**, $*p < 0.05$, $****p < 0.0001$, and d vs the ACTH + VEH treatment group in **B**, $##p < 0.01$ and d vs the VEH + VEH treatment group in **C**, and $**p < 0.01$, $***p < 0.001$ and d vs the ACTH + VEH treatment group in **D**. ACTH: adrenocorticotrophic hormone. d : unbiased Cohen's d -value. ESC-15: escitalopram oxalate (15 mg/kg). IMI-15: imipramine hydrochloride (15 mg/kg). KYNUA: kynurenic acid. QUINA: quinolinic acid. SIL-3: sildenafil citrate (3 mg/kg). SIL-10: sildenafil citrate (10 mg/kg). VEH: vehicle control.

showed, all in ACTH-treated rats, that sub-acute IMI-15 ($p < 0.0001$, $d = 1.76$), ESC-15 ($p < 0.0001$, $d = 1.69$), SIL-3 ($p = 0.0457$, $d = 0.65$), SIL-10 ($p < 0.0001$, $d = 2.05$), IMI-15 + SIL-3 ($p < 0.0001$, $d = 1.28$), IMI-15 + SIL-10 ($p < 0.0001$, $d = 1.25$) and ESC-15 + SIL-3 ($p < 0.0001$, $d = 1.60$) treatments significantly reduce hippocampal KYNUA levels compared to VEH-treated rats (see Fig. 6B).

An unpaired Student's t -test revealed a statistically significant

difference between the ACTH + VEH and the VEH + VEH control group regarding hippocampal QUINA levels. Therefore, sub-chronic ACTH treatment significantly elevates hippocampal QUINA levels compared to VEH-treated SD rats ($p = 0.0052$, $d = 1.22$) (see Fig. 6C). A Kruskal-Wallis one-way ANOVA test for multiple comparisons, all in ACTH-treated rats, indicated statistically significant differences between the various sub-acute treatment groups regarding hippocampal QUINA

levels ($p = 0.0017$). Dunn's post-hoc test for multiple comparisons showed, all in ACTH-treated rats, that sub-acute IMI-15 ($p = 0.0025$, $d = 1.49$), SIL-10 ($p = 0.0002$, $d = 1.61$) and IMI-15 + SIL-3 ($p = 0.0044$, $d = 1.36$) treatments significantly reduce hippocampal QUINA levels compared to VEH-treated rats (see Fig. 6D).

3.2.5. Hippocampal glutathione and glutathione disulfide levels

Fig. 7 below depicts the total hippocampal glutathione and

glutathione disulfide levels of SD rats after receiving sub-chronic VEH or ACTH treatment, followed by sub-acute VEH or drug treatment.

A Mann-Whitney U test revealed a statistically significant difference between the ACTH + VEH and the VEH + VEH control group regarding hippocampal glutathione levels. Therefore, sub-chronic ACTH treatment significantly elevates hippocampal glutathione levels compared to VEH-treated SD rats ($p < 0.0001$, $d = 2.71$) (see Fig. 7A). A Kruskal-Wallis one-way ANOVA test for multiple comparisons, all in ACTH-treated

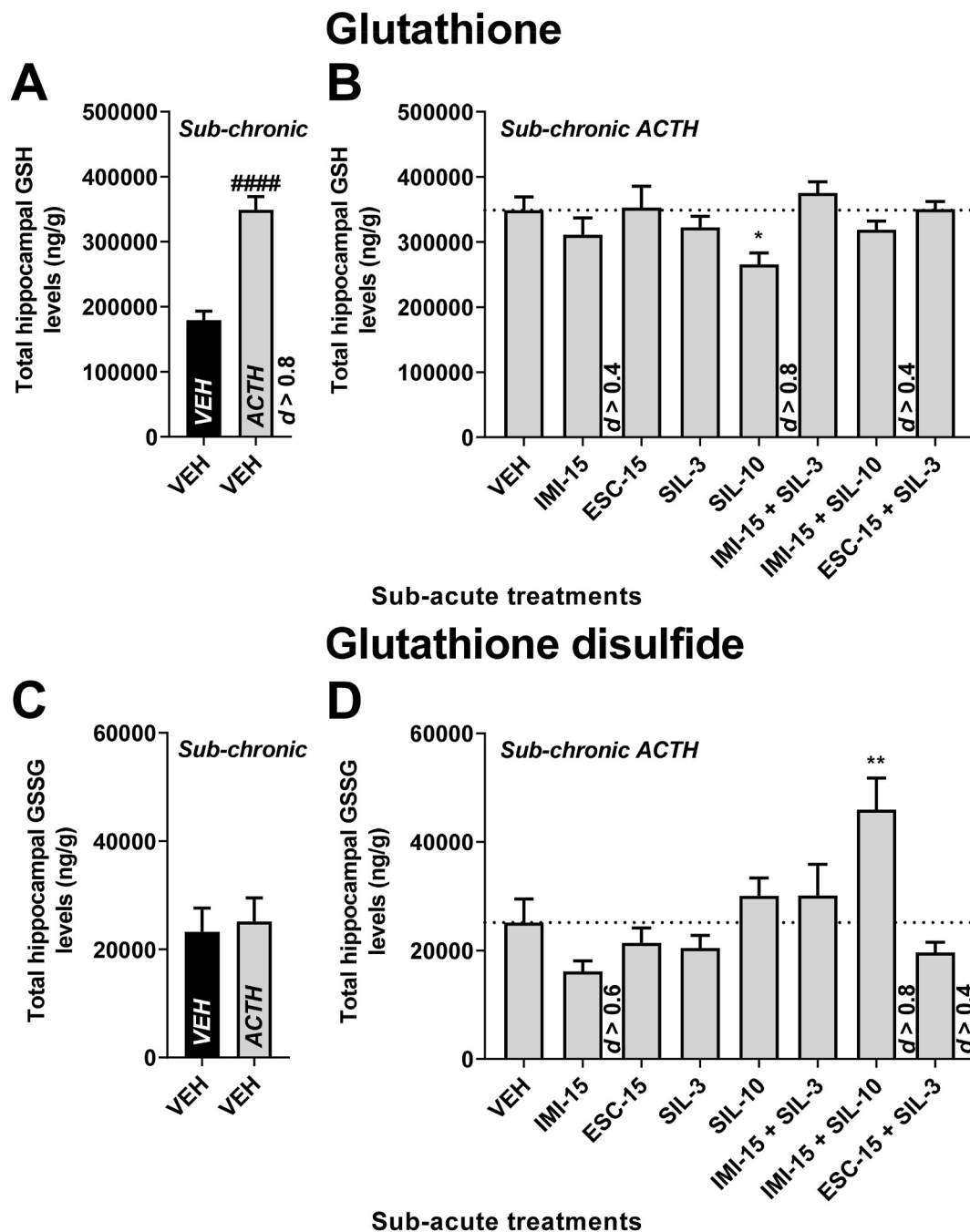


Fig. 7. A graphical representation of the hippocampal glutathione and glutathione disulfide assay data. **A** Glutathione levels of rats after receiving sub-chronic VEH or ACTH treatment, followed by sub-acute VEH treatment. **B** Glutathione levels of rats after receiving sub-chronic ACTH treatment, followed by sub-acute VEH or drug treatment. **C** Glutathione disulfide levels of rats after receiving sub-chronic VEH or ACTH treatment, followed by sub-acute VEH treatment. **D** Glutathione disulfide levels of rats after receiving sub-chronic ACTH treatment, followed by sub-acute VEH or drug treatment. All group sizes are equal ($n = 12$). Data points represent the mean $\pm$ SEM. Statistical analyses are reported in the text with #### $p < 0.0001$ and d vs the VEH + VEH treatment group in **A**, * $p < 0.05$ and d vs the ACTH + VEH treatment group in **B**, and ** $p < 0.01$ and d vs the ACTH + VEH treatment group in **D**. ACTH: adrenocorticotrophic hormone. d : unbiased Cohen's d -value. ESC-15: escitalopram oxalate (15 mg/kg). IMI-15: imipramine hydrochloride (15 mg/kg). SIL-3: sildenafil citrate (3 mg/kg). SIL-10: sildenafil citrate (10 mg/kg). VEH: vehicle control.

rats, indicated statistically significant differences between the various sub-acute treatment groups regarding hippocampal glutathione levels ($p = 0.0016$). Dunn's post-hoc test for multiple comparisons showed, all in ACTH-treated rats, that sub-acute SIL-10 ($p = 0.0125$, $d = 1.21$) treatment significantly reduces hippocampal glutathione levels compared to VEH-treated rats (see Fig. 7B).

A Mann-Whitney U test revealed no statistically significant difference between the ACTH + VEH and the VEH + VEH control group regarding hippocampal glutathione disulfide levels ($p = 0.5899$, $d = 0.12$) (see Fig. 7C). An Ordinary one-way ANOVA test for multiple comparisons, all in ACTH-treated rats, indicated statistically significant differences between the various sub-acute treatment groups regarding hippocampal glutathione disulfide levels ($F_{(7, 88)} = 6.091$, $p < 0.0001$). Dunnett's post-hoc test for multiple comparisons showed, all in ACTH-treated rats, that sub-acute IMI-15 + SIL-10 ($p = 0.0015$, $d = 1.13$) treatment significantly elevates hippocampal glutathione disulfide levels compared to VEH-treated rats (see Fig. 7D).

4. Discussion

In this study, the ACTH-induced rodent model of TRD induced significant antidepressant-like effects together with noteworthy changes in certain depression-related biomarkers. However, the latter were for the most part unresponsive to sub-acute treatments with IMI-15, ESC-15, SIL-3, and IMI-15 + SIL-10, but responsive to SIL-10, IMI-15 + SIL-3, and ESC-15 + SIL-3.

Sub-chronic ACTH treatment had no significant impact on locomotor activity (Fig. 2A), consistent with earlier studies (Kitamura et al., 2002; Walker et al., 2013; Pereira et al., 2019). In ACTH-treated rats, sub-acute IMI-15 and ESC-15 did not significantly alter locomotor activity (Fig. 2B), but IMI-15 exhibited a tendency toward reduced activity ($d = 1.02$). Previous studies in ACTH-naïve rats reported variable effects for imipramine (Liebenberg et al., 2010b; Guan et al., 2014) and negligible effects for escitalopram (Mokoena et al., 2015). Furthermore, SIL-3 and SIL-10 did not significantly alter locomotor activity (Fig. 2B), consistent with prior research (Liebenberg et al., 2010a, 2010b; Saayman et al., 2021). However, IMI-15 + SIL-3 and IMI-15 + SIL-10 significantly reduced locomotor activity, suggesting that both doses of sildenafil augmented the effect of IMI-15 on locomotor activity.

Following ANCOVA adjustments for locomotor activity, immobility in Fig. 2C and D reflects psychomotor activity, i.e., depressive-like behaviour. ACTH significantly increased depressive-like behaviour (time spent immobile) in the FST compared to VEH-treated animals (Fig. 2C), indicating a causal role for robust activation of the hypothalamic-pituitary-adrenal (HPA) axis, as seen in MDD (Pariante and Lightman, 2008; Baumeister et al., 2016) and TRD (Jurueña et al., 2013; Markopoulou, 2013). Nevertheless, similar studies have failed to show exaggerated depressive-like behaviour following ACTH treatment (Walker et al., 2013; Pereira et al., 2019). As expected, IMI-15 and ESC-15 did not significantly alleviate depressive-like behaviour in ACTH-treated rats (Fig. 2D), confirming that the model exhibits treatment resistance to at least two classes of monoaminergic active antidepressants, aligned with previous studies (Walker et al., 2013; Antunes et al., 2015; Pereira et al., 2019).

SIL-10, but not SIL-3, significantly reduced depressive-like behaviour in ACTH-treated rats (Fig. 2D), not only reaffirming sildenafil's previously demonstrated antidepressant properties (Brink et al., 2008; Wang et al., 2014; Saayman et al., 2021), but also highlighting for the first time its ability to overcome antidepressant resistance in a dose-dependent manner. Furthermore, that IMI-15 + SIL-3 and ESC-15 + SIL-3 significantly reduced depressive-like behaviour, where either antidepressant as monotherapy was ineffective, demonstrates that low-dose sildenafil can augment the activity of traditional antidepressants. IMI-15 (having antimuscarinic properties) + SIL-10 treatment, unlike either drug alone, showed a notable trend toward reducing depressive-like behaviour ($d = 0.85$) (Fig. 2D). Only higher doses of sildenafil (≥ 10 mg/kg) have been

demonstrated before to require an antimuscarinic agent to reveal an antidepressant-like effect in a rodent depression model (Brink et al., 2008; Liebenberg et al., 2010a), which is not a finding replicated in the ACTH model of TRD.

Despite inducing a depressive-like state (Fig. 2C), ACTH did not significantly raise plasma corticosterone levels (Fig. 3A). Others observed a transient increase in plasma corticosterone by ACTH, peaking on day 4 and returning to normal levels afterward, suggesting HPA system down-regulation (Walker et al., 2013). Clinical data (Carroll et al., 2007) supports the idea that elevated ACTH secretion typically does not lead to long-term hypercortisolaemia (Walker et al., 2013). Nevertheless, ACTH caused a trend toward increased plasma corticosterone levels, with a medium effect size ($d = 0.61$) (Fig. 3A), as reported before (Pereira et al., 2019). Our findings confirm that ACTH causes a sustained depressive-like state in rats resistant to antidepressant treatment. HPA axis hyperactivity and impaired negative feedback regulation are strongly linked to MDD and TRD (Barden, 2004; Boyle et al., 2005; Anacker et al., 2011; Baumeister et al., 2016), with chronic antidepressant treatment downregulating the HPA axis (Satoh et al., 1985; Farahbakhsh and Radahmadi, 2022) as a critical drug target (Frost et al., 2003).

IMI-15 and ESC-15 failed to significantly reduce plasma corticosterone levels (Fig. 3B), possibly contributing to the treatment resistance. Similar results have been reported before for amitriptyline and imipramine (Antunes et al., 2015), whereas Bano et al. (2010) reported conflicting results for antidepressants, e.g., that sertraline and moclobemide had no significant effect on corticosterone, while citalopram reduced corticosterone, and tianeptine increased them (Bano et al., 2010). Sub-acute SIL-3, SIL-10, IMI-15 + SIL-3, and IMI-15 + SIL-10 treatments also failed to significantly affect plasma corticosterone levels in ACTH-treated rats (Fig. 3B). Importantly, despite some evidence for drug-induced changes in corticosterone, i.e., ESC-15 + SIL-3 treatment reducing its levels, corticosterone was not elevated in ACTH-treated rats; a study limitation that complicates the interpretation of treatment effects on corticosterone, warranting further research. Consequently, no precise and reliable interpretation of treatment-related effects in ACTH-treated rats is possible.

ACTH unexpectedly raised hippocampal BDNF levels (Fig. 4A), contrary to the typical association of reduced BDNF levels with MDD (Lee and Kim, 2010), TRD (Li et al., 2007), and HPA axis hyperactivity (Jacobsen and Mørk, 2006; Huang et al., 2011; Gong et al., 2016). That said, IMI-15 and ESC-15 reduced this elevation in hippocampal BDNF levels in ACTH-treated rats (Fig. 4B). While these findings oppose the negative neurotrophic hypothesis of MDD, which links BDNF reversal with antidepressant efficacy (Duman and Monteggia, 2006; Polyakova et al., 2015; Duman et al., 2016), there are considerations to keep in mind. Changes in BDNF may be related to co-presenting metabolic and redox factors, with BDNF potentially exerting counter-regulatory effects on glutathione oxidation or mediating redox effects itself, contributing to mood disorder development (Harvey et al., 2012; Brand et al., 2015). Yet sub-acute SIL-10, IMI-15 + SIL-3, IMI-15 + SIL-10, and ESC-15 + SIL-3 treatments all significantly reduced hippocampal BDNF levels in ACTH-treated rats, with sub-acute SIL-3 showing a trend toward reduction ($d = 0.89$) (Fig. 4B). The counter-regulatory role of BDNF may be dependent on co-morbid redox and inflammatory conditions (Harvey et al., 2012). Importantly, these states may reflect illness severity, and in turn response to antidepressants (Mokoena et al., 2010, 2015). Yet, it is unlikely that the antidepressant-like effects induced by sildenafil in the FST are solely mediated by a downregulation in BDNF signalling, considering the same reduction in the hippocampal levels of BDNF was observed following sub-acute IMI-15, ESC-15, and IMI-15 + SIL-10 treatments, which failed to reduce the immobility evident in ACTH-treated rats.

ACTH significantly raised hippocampal serotonin and norepinephrine levels in rats (Fig. 5A and C, respectively), contrary to predictions of the biogenic amine hypothesis of MDD (Brand et al., 2015). Others have

also reported increased prefrontocortical and hippocampal serotonin and norepinephrine levels in rats after ACTH treatment (Li et al., 1990; Walker et al., 2013). Notably, IMI-15 and ESC-15 reduced elevated hippocampal serotonin levels in ACTH-treated rats while leaving norepinephrine levels unaffected (Fig. 5B and D). Interestingly, SIL-3 and SIL-10 significantly lowered norepinephrine levels (Fig. 5D), with ESC-15 + SIL-3 reducing only hippocampal serotonin levels (Fig. 5B) and showing a trend toward reducing norepinephrine levels ($d = 0.67$) (Fig. 5D). However, IMI-15 + SIL-3 and IMI-15 + SIL-10 did not significantly impact hippocampal serotonin and norepinephrine levels (Fig. 5B and D), with a trend toward reducing serotonin levels ($d = 0.62$ and $d = 0.70$, respectively). Unlike that observed in ACTH-treated SD rats, previous reports suggest that sildenafil partly exerts its antidepressant-like effects in Flinders Sensitive Line (FSL) rats, i.e., an animal model of MDD, by modulating the monoaminergic system, particularly enhancing serotonergic and/or noradrenergic neurotransmission (Liebenberg et al., 2010a, 2010b). The reduction in time spent immobile in the FST induced by sildenafil can also not be fully explained by reduced hippocampal serotonergic and noradrenergic levels, since this was not consistently demonstrated across the various sildenafil mono- and combination therapies. Multimodal neurobiological mechanisms appear to be involved.

Based on the above findings, we conclude that the neurobiological profile of TRD may differ from that of MDD, which is not surprising since TRD resists monoaminergic active antidepressants. Thus, the ACTH-induced rodent model of TRD exhibits elevated hippocampal BDNF levels, whereas genetic rodent models of MDD, like the FSL rat, demonstrate the opposite (Elfving et al., 2010; Overstreet and Wegener, 2013). Both models, however, share increased hippocampal serotonin and norepinephrine levels, as observed here and in other studies (Zangen et al., 1997, 1999). ACTH may, therefore, counteract the effects of monoaminergic antidepressants by blocking their primary mechanism of action, thereby failing to elevate central synaptic monoaminergic and subsequently BDNF levels.

SIL-10, IMI-15 + SIL-3, and ESC-15 + SIL-3 may induce their antidepressant-like effects (in the FST) independently of the monoaminergic system and BDNF signalling pathway, explaining sildenafil's effectiveness in a rodent model resistant to monoaminergic antidepressants. In fact, the antidepressant-like action of PDE5 inhibitors may partially depend on PDE5 inhibition and subsequent cGMP signalling (Brink et al., 2008; Liebenberg et al., 2010a). cGMP modulates various monoamine transmitter systems (Harvey, 1996), along with roles in inflammation (Raposo et al., 2014; Nguyen et al., 2022), oxidative stress (Masood et al., 2008; Curatola et al., 2011), and neuroplasticity (Gallo and Iadecola, 2011; Reiersen et al., 2011), all implicated in MDD and antidepressant action (Brand et al., 2015).

That said, the tryptophan-kynurenine pathway may serve as a connecting mechanism. Current MDD literature suggests a shift in tryptophan metabolism away from serotonin, playing a vital role in mood regulation (Marx et al., 2021; Hestad et al., 2022), specifically towards the kynurenine pathway (Brand et al., 2015) which is involved in neuroinflammation via glutamatergic actions (Moller et al., 2015). Elevated indoleamine-2,3-dioxygenase (IDO), as activated by pro-inflammatory cytokines, depletes tryptophan and its derived neurotransmitters like serotonin, while increasing downstream kynurenine metabolites, such as neuroprotective KYNUA and neurotoxic QUINA. The KYNUA:QUINA ratio determines a subtle balance between neuroprotection and neurotoxicity (Brown et al., 2021; Marx et al., 2021). In this study, ACTH significantly increased hippocampal KYNUA and QUINA levels (Fig. 6A and C), which may indicate maintenance of a neuroprotective balance (Moller et al., 2015). MDD is often associated with a shift in kynurenine metabolism towards QUINA, especially in cases of hippocampal shrinkage (Savitz et al., 2015; Marx et al., 2021; Öztürk et al., 2021; Hestad et al., 2022). Importantly, antidepressants can reverse or stabilize this shift (Halaris et al., 2015; Sun et al., 2020; Ou et al., 2023).

IMI-15 and ESC-15 significantly reduced elevated hippocampal

KYNUA levels, whereas IMI-15 but not ESC-15 reduced QUINA (Fig. 6B and D). Contrary to some of our findings, monoaminergic antidepressants usually reduce QUINA levels and increase KYNUA levels in MDD patients (Halaris et al., 2015; Kocki et al., 2018). Our findings in the ACTH model of TRD suggests a unique mechanism that might be specific to this model. SIL-3, SIL-10, IMI-15 + SIL-3, IMI-15 + SIL-10, and ESC-15 + SIL-3 significantly reduced hippocampal KYNUA levels (Fig. 6B), whereas SIL-10 and IMI-15 + SIL-3 significantly reduced hippocampal QUINA levels. Notably, SIL-3, IMI-15 + SIL-10, and ESC-15 + SIL-3 induced large effect size reductions in hippocampal QUINA levels (Fig. 6D). Sildenafil-induced elevations in the hippocampal levels of KYNUA (neuroprotective) therefore do not appear to underlie its antidepressant-like effects in ACTH-treated rats. Moreover, although reduced QUINA levels, and thus a reduction in its neurotoxic effects, may play a role in the mechanism of action of sildenafil, the failure of sildenafil mono- and combination therapies to consistently reduce QUINA levels in the ACTH model of TRD, while IMI-15 was able to reduce said levels as well as evoke antidepressant effects, suggest that sildenafil does not induce its antidepressant-like effects solely by reducing QUINA.

That ACTH significantly raised hippocampal glutathione levels, without effect on glutathione disulfide (Fig. 7A and C), contrasts with oxidative stress and reduced antioxidant, i.e., glutathione, levels associated with MDD (Gawryluk et al., 2011; Freed et al., 2017; Song et al., 2021). However, protective mechanisms may be at play, initially boosting state-dependent antioxidant processes to combat oxidative stress. IMI-15 and ESC-15 did not affect hippocampal glutathione and glutathione disulfide levels (Fig. 7B and D), whereas SIL-10, but not SIL-3, IMI-15 + SIL-3, IMI-15 + SIL-10, and ESC-15 + SIL-3 significantly reduced hippocampal glutathione levels (Fig. 7B). These findings suggest that the antidepressant-like effects of sildenafil mono- and combination therapy cannot be ascribed exclusively to its antioxidant properties, i.e., ability to increase glutathione levels.

In summary, the ACTH model has demonstrated treatment resistance to IMI-15 and ESC-15 while the antidepressant-like effects of sildenafil, whether used alone or as an augmentation strategy, appear to be dose-dependent. A higher/optimal dose of sildenafil (10 mg/kg) is needed as monotherapy to overcome antidepressant treatment resistance in this model, while a low dose (3 mg/kg) augments the effects of co-administered monoaminergic antidepressants (IMI-15 and ESC-15). Hippocampal BDNF, serotonin, norepinephrine, KYNUA, QUINA, and GSH levels were significantly elevated in ACTH-treated animals compared to controls, but plasma corticosterone remained unchanged in the model. It may be that the sustained effects on BDNF, serotonin, norepinephrine, KYNUA, QUINA, and glutathione levels were initially induced and sustained by elevated corticosterone early on in ACTH treatment. The effects of corticosterone eventually dissipated, representing an immediate early-gene-related event that switches on genes whose post-transcriptional effects are felt for a prolonged period even though corticosterone has long since been down-regulated. IMI-15 and ESC-15, as well as SIL-3 and SIL-10, and IMI-15 + SIL-3, IMI-15 + SIL-10, and ESC-15 + SIL-3, reversed some of these changes. Concluding, sildenafil is therefore a typical example of a multimodal antidepressant, although further studies are needed to unravel the exact mechanisms of its promising antidepressant and augmentative effects.

5. Ethical standards

All efforts were made to minimise animal suffering during this study. Ethical approval was given as described under materials and methods above. Housing conditions and all experiments and procedures performed as part of this study complied with the institutional policies and other guidelines on the care and use of laboratory animals and national legislation. The guidelines of the South African National Standards: The Care and Use of Animals for Scientific Purposes (SANS 10386:2008) also informed how animals were kept and the way experiments and

procedures were carried out, as well as the Ethics in Health Research: Principles, Processes and Structures guidelines of 2015.

Smith et al. (2018) have recently developed a set of planning guidelines to improve the conduction, reporting, and appraisal of animal research, referred to as Planning Research and Experimental Procedures on Animals: Recommendations for Excellence (PREPARE) guidelines (Smith et al., 2018). As such, the PREPARE guidelines were carefully considered and implemented during the planning phase of this study to enhance the quality, reproducibility, and translatability of experimental results. All experimental data are reported according to the National Centre for the Replacement, Refinement, and Reduction of Animals in Research's (NC3Rs) Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines to promote a transparent, reproducible, comprehensive, accurate, concise, logically ordered, and well-written manuscript (Kilkenny et al., 2010).

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6. Statement of interest

GW reported having received research support/lecture/consultancy fees from H. Lundbeck A/S, Eli Lilly A/S, Takeda/Shire A/S, HB Pharma A/S, Arla Foods Amba., Janssen Pharma A/S and Mundipharma International, Ltd.

BHH and CBB declare that, except for income from the primary employer (NWU) and research funding to JLBS (SAAWK; Jaschafoundation), these organisations did not have any vested interest in the study. BHH has participated in advisory boards and received honoraria from Adcock-Ingram, Servier and Lundbeck, and has received research funding from Servier, Lundbeck, and HG&H Pharma. No other financial support or compensation has been received from any individual or corporate entity over the past three years for research or professional services, and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

CRedit authorship contribution statement

Juandré Lambertus Bernardus Saayman: Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation. **Brian Herbert Harvey:** Writing – review & editing, Validation, Supervision, Methodology, Funding acquisition, Conceptualization. **Gregers Wegener:** Writing – review & editing, Validation, Methodology, Funding acquisition, Data curation. **Christiaan Beyers Brink:** Writing – review & editing, Visualization, Validation, Supervision, Data curation, Conceptualization.

Declaration of competing interest

Gregers Wegener reported having received research support/lecture/consultancy fees from H. Lundbeck A/S, Eli Lilly A/S, Takeda/Shire A/S, HB Pharma A/S, Arla Foods Amba., Janssen Pharma A/S and Mundipharma International, Ltd.

Data availability

Data will be made available on request.

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