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Research Articles

Cortisol-dependent impairment of dendrite plasticity in human dopaminergic neurons derived from hiPSCs is restored by ketamine: Relevance for major depressive disorders.

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ABSTRACT

Impaired neuroplasticity in neurons endowed in limbic circuits is considered a hallmark of chronic stress and depression. The reasons for this impairment are still partially unclear, but converging findings suggest that it can be reverted by exposure to rapid-acting antidepressants. In this study we revamped the hypothesis that the abnormal high circulating levels of cortisol observed in Major Depressive Disorders with anhedonia may contribute to drive the limbic circuit neuroplasticity impairment. Here we used an established in-vitro translational model based on human iPSC-derived dopaminergic neurons to extend the evidence obtained in rodents of glucocorticoid-induced hypotrophy of cortical dendrites. The predictive value of this model was tested by assessing the reversal potential of rapid-acting antidepressants on cortisol-induced hypotrophy. Human mesencephalic dopaminergic neurons were differentiated in-vitro from healthy donor iPSCs for 60–70 days. Cortisol effects were assessed by measuring maximal dendrite length, primary dendrite number and soma area 3 days after last exposure. Concentration- and time-response curves were initially established. Cortisol produced a concentration- and time-dependent reduction of dendritic arborization of human dopaminergic neurons, with maximal effects at 50 μM for 4-day dosing. These effects were reverted when followed by 1-hr exposure to ketamine or (2R,6R)-hydroxynorketamine at concentrations of 0.01 μM and 0.05 μM, respectively, resulting approximately 10- or 100-fold lower than those effective in neurons not exposed to cortisol. Overall, in this study high cortisol impaired dendritic arborization in human dopaminergic neurons and sensitized their neuroplasticity response to very low doses of rapid-acting antidepressants known to upregulate AMPA-mediated glutamatergic neurotransmission.

1. Introduction

Hypercortisolemia is one of the biological hallmarks of Major Depression Disorder (MDD) (Deuschle et al., 1998; Gold and Chrousos, 2002; Nikkheslat et al., 2020). Cortisol is a circulating glucocorticoid secreted by the adrenal cortex under the hypothalamic-pituitary (HPA) control, characterized by a circadian rhythm upon which peaks of transient increases occur during stress responses. In most patients with MDD the circadian profile is suppressed, and the average levels are chronically increased, i.e., 2–3 folds the normal values, indicating the HPA hyperactivity (Gold and Chrousos, 2002; Pariante and Lightman, 2008; Binder et al., 2009). Chronically elevated cortisol levels are associated with poorer outcomes in the treatment of MDD (Strawbridge et al., 2019), suggesting a possible role in determining the Treatment Resistant Depression (TRD) condition.

From the pathophysiological standpoint, stress precipitates symptomatic depression via a complex peripheral/central interaction that involves the HPA axis, the autonomic nervous system and several central limbic structures whose neurons express glucocorticoid receptors (McEwen and Gianaros, 2011; Gold et al., 2015; Anderson et al., 2016, Barfield and Gourley, 2018). Intriguingly, in rodents, exposure to glucocorticoids or chronic stress produces hypotrophic effects in limbic

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circuits, as exemplarily showed by the reduced dendrite arborization of neurons of the limbic prefrontal cortex and hippocampus (Watanabe et al., 1992, Krishnan and Nestler, 2008, McEwen and Gianaros, 2011, Liston and Gan 2011, Gold et al., 2015, Anderson et al., 2016, Yau et al., 2016, Barfield and Gourley, 2018). In a series of studies, these chronic stress-dependent hypotrophic effects in neurons were counteracted by ketamine, a rapid-acting antidepressant that engages the BDNF-dependent Akt/ERK-mTORC1 pathway via glutamatergic transmission, known to be critically involved in the structural plasticity of dendritic arborization (Jourdi et al., 2009; Li et al., 2010, Autry et al., 2011, Lepack et al., 2014; Krystal et al., 2013, Björkholm and Monteggia, 2016). Low doses of ketamine as bolus infusion were associated to behavioral and symptomatic improvements in stress-exposed rodents and in humans with diagnosis of MDD/TRD with effects lasting up to one week (Zarate et al., 2006, Koike et al., 2011; Krystal et al., 2013, Coyle and Laws, 2015), providing a strong rationale for linking neuroplasticity-related changes in limbic neurons and antidepressant effects. This appears of relevance for TRD patients, whose lack of response to SSRI could be related to a more resilient neuroplasticity impairment in limbic circuits (Mathew et al., 2012; Serafini et al., 2014; Fava et al., 2020).

Among these limbic circuits, the dopaminergic (DA) mesencephalic system is considered a relevant substrate for the MDD core symptoms of anhedonia and low energy (Pizzagalli, 2014; Barik et al., 2013). A recent [18F]-DOPA imaging study showed that subjects with an history of chronic high psychosocial stress, when exposed to an acute social stressor, displayed a reduced striatal DA synthesis when compared to subjects with an history of low psychosocial stress (Bloomfield et al., 2019). These data are in line with the evidence that chronic stress in rodents significantly attenuates the release of dopamine from DA neurons of the mesolimbic system (Gambarana et al., 1999; Shimamoto et al., 2011). Cellular studies on the mesencephalic DA system indicate that acute and chronic stress produce neuroadaptive functional changes, for example affecting the AMPA receptors functioning in DA neurons (Belujon and Grace, 2014; Holly and Miczek 2016). Depressive-like symptoms and increased circulating glucocorticoids were also measured during withdrawal from addictive drugs, phenomena associated with impaired neuroplasticity of the mesocorticolimbic DA system (Nestler 2013; Koob and Volkow 2016). However, direct evidence of such modulatory role of glucocorticoids on human DA neurons are not available.

We propose to approach this problem by excploring a relatively new *in vitro* pharmacological paradigm, i.e., implementing human DA neurons differentiated from inducible pluripotent stem cells (iPSCs) obtained from consenting donors (Kriks et al., 2011; Fedele et al., 2017; Collo et al., 2018a; Polit et al., 2023). In recent studies, structural plasticity driven by various pharmacological agents was investigated measuring changes in dendrite arborization and soma size three days after drug challenge in two well-characterised iPSC from healthy donors (Collo et al. 2018a, 2018b). In particular, the glutamatergic antidepressants ketamine and its active metabolite (2R,6R)-hydroxynorketamine (HNK) (Zanos et al., 2016) were demonstrated to enhance dendritic arborization and soma size in human DA neurons differentiated form iPSC when exposed for 1-hr (Cavalleri et al., 2018; Collo et al., 2018c), at concentrations in the high-range of those measured *in vivo* in rodents and in human clinical trials (Zanos et al., 2016; Zhao et al., 2012).

In this study the putative hypotrophic effect of cortisol on dentrite arborization of human DA neurons will be initially investigated by characterizing its concentration- and time-dependency. The initial ranges of concentration and exposure time will be mutuated from the literature in which human iPSC-derived brain cells or primary telencephalic cultures from rodents were exposed to glucocorticoids *in vitro* (Heard et al., 2021; Cruceanu et al., 2022; Bassil et al., 2023), here expressed as cortisol-equivalents being the various glucocorticois characterized by different potencies (Nicolaides et al., 2000). Once the most effective concentration and exposure time will be identified, various concentrations of ketamine or HNK will be used to restore the dendrite arborization to normal, when compared with vehicle-exposed neuronal cultures. The rationale for defining the concentration range of ketamine and HNK was based on previous *in vitro* studies on human DA neurons (Cavalleri et al., 2018) and on data from human pharmacokinetic studies reported in patients with mood disorders (Zhao et al., 2012), so to provide a translational linkage for the result interpretation.

2. Methods

2.1. Pharmacological agents

In the present study the following pharmacological agents were used: Hydrocortisone (cortisol) (Tocris Bioscience, Bristol, UK), Ketamine (ACME, Reggio Emilia, Italy), HNK (Tocris Bioscience). For each vehicle treatment, the solvents required by the specific drugs were used at the same dilution as for the active treatment.

2.2. Differentiation of human iPSCs into midbrain DA neuron phenotype

Human iPSC (hiPSC) clone F3 previously generated and characterized (Collo et al., 2018a) was induced to differentiate into floorplate (FP)-derived midbrain DA neurons using dual-SMAD inhibition and FP induction (Kriks et al., 2011; Fedele et al., 2017). HiPSCs were dissociated with Accutase™ (StemCell Technologies, Vancouver, BC, Canada), seeded (3×10^4 cells/cm^2) on Matrigel-coated plates in Knockout Serum Replacement (KSR) medium containing Knockout™ DMEM, 15% KSR, GlutaMAX™ and 10 μM 2-mercaptoethanol, in the presence of LDN193189 (0.1 μM, Stemgent, Cambridge, MA), SB431542 (10 μM, Tocris Bioscience), Shh C25II (0.1 μg/ml, R&D Systems), Purmorphamine (2 μM, Stemgent), Fibroblast Growth Factor 8 (0.1 μg/ml, R&D Systems) and CHIR99021 (3 μM, Stemgent). From day 5, KSR medium was shifted to N2 medium (Knockout™ DMEM/F12, N2 supplement and GlutaMAX™, all from Gibco-Invitrogen). On day 11, the medium was changed to Neurobasal/B27/GlutaMAX™ supplemented with CHIR99021, BDNF (20 ng/ml, R&D Systems), ascorbic acid (AA; 0.2 mM, Sigma-Aldrich), dibutyryl cAMP (cAMP; 0.5 mM, Sigma-Aldrich), transforming growth factor type β3 (TGFβ3; 1 ng/ml, R&D Systems), glial cell line-derived neurotrophic factor (GDNF; 20 ng/ml, R&D Systems) and DAPT (10 nM, Tocris Bioscience). On day 21, cells were seeded on plates pre-coated with Polyornithine/Fibronectin/Laminin and co-cultured with mouse primary cortical astrocytes (Collo et al., 2018a). Pharmacological treatments were performed from day 50. At this stage DA neurons co-expressed tyrosine hydroxylase (TH) and MAP2 (Collo et al., 2018a). At this stage, TH+/MAP2+ DA neurons accounted for 30% of the total MAP2+ neurons, while the other neuronal populations consisted of GABAergic (20–25%) or glutamatergic (35–40%) neurons (Collo et al., 2018a; Cavalleri et al., 2018).

2.3. In vitro pharmacological experiments

In all pharmacological experiments, 4 days before treatments (Day −4), the culture medium of differentiated DA neurons was progressively deprived of BDNF, AA, cAMP, TGFβ3, GDNF and DAPT and replaced with Neurobasal/B27 supplement (by −50% each day). The experiment aimed to characterize the matrix of cortisol dose-time response curves by evaluating changes in the morphology (Fig. 1A). Briefly, at Day 0 neuronal cultures were randomly allocated to 4 groups of treatments, consisting of either vehicle or cortisol (10 μM, 25 μM and 50 μM). Each group was subdivided into another 4 groups and exposed to cortisol for different periods, i.e., 24hr, 48hr, 72hr and 96hr (from day 1 to day 4) before cell culture medium replacement. All cell cultures underwent fixation with paraformaldehyde 4% for immuno-cytochemistry at 72 h after last treatment (Day 7), as previously described (Collo et al., 2018a; Cavalleri et al., 2018). Each treatment group was assessed in duplicate

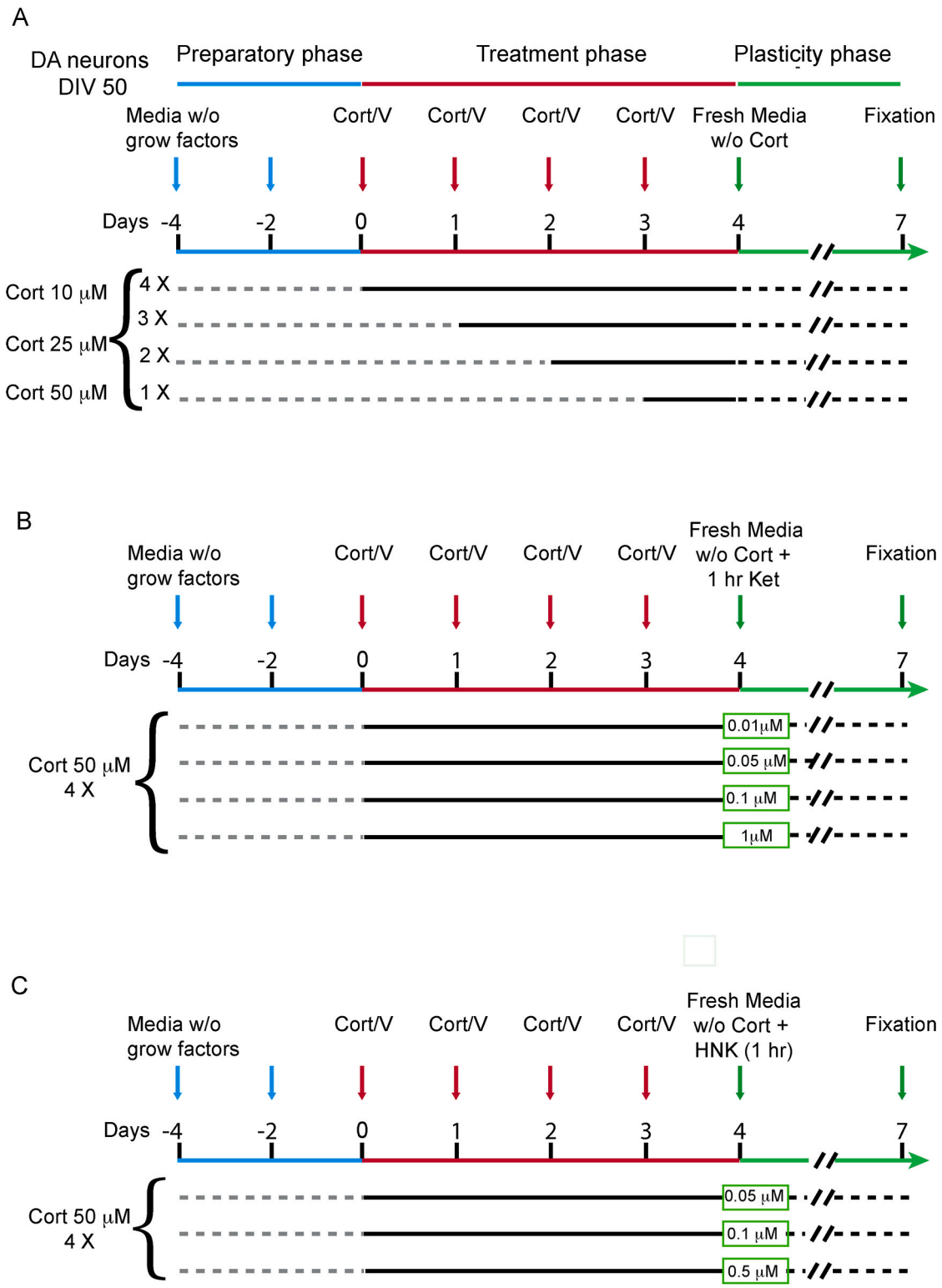

Fig. 1. Schematic representation of the experimental protocols. (A) Characterization of the cortisol concentrations and exposure time required to produce hypothrophic effects on hiPSC-derived DA neurons. A matrix design was used, attributing each of the 3 predefined concentrations of cortisol (10, 25 and 50 μM) to 4 different exposure time (1, 2, 3 and 4 days). The Preparatory phases consisted of 4 days, while the Plasticity phase, i.e., necessary to assess the neuroplasticity response to treatments, required 3 days, all in common with the various conditions. **(B)** Assessment of the concentration-response curve of the restoring properties of ketamine (0.01–1 μM) on DA neurons previously exposed to the most effective dose of cortisol obtained in (A). Exposure time was fixed at 1 h, as previously shown (Cavalleri et al., 2018). The Plasticity phase, ending with Fixation of the cultures, was the same as described in (A). **(C)** Assessment of the concentration-response curve of the restoring properties of HNK (0.05–0.5 μM) on DA neurons previously exposed to the most effective dose of cortisol obtained in (A). Exposure time was fixed at 1 h, as previously shown (Cavalleri et al., 2018). The Plasticity phase, ending with Fixation of the cultures, was the same as described in (A). Cor: cortisol; V: vehicle; Ket: ketamine; HNK: (2R,6R)-hydroxynorketamine.

coverslides. Each concentration- and time-response experiment was repeated at least three times.

The second experiment aimed to investigate the effect of ketamine in counteracting the effects of cortisol on human DA neurons by evaluating changes in morphology (Fig. 1B). Human DA neurons exposed to the most effective concentration x time conditions of cortisol as defined in the first experiment, were challenged with various concentration of ketamine (0.01, 0.05, 0.10, 1 μM) or vehicle for 1h before cell culture media replacement. As in the first experiment, cell cultures were fixed with paraformaldehyde 4% at 72 h after last treatment (Day 7). Each treatment group was assessed in duplicate coverslides. Each experiment was repeated at three times.

The third experiment was aimed to study the effect of HNK to counteract the effects of cortisol on human DA neurons by assessing changes in morphology (Fig. 1C). Human DA neurons, exposed to the most effective concentration x time conditions of cortisol as defined in the first experiment, were challenged with various concentration of HNK (0.05, 0.10, 0.5 μM) or vehicle for 1h before cell culture media replacement. As shown in the first and second experiments, cell cultures were fixed with paraformaldehyde 4% at 72 h after last treatment (Day 7). Each treatment group was assessed in duplicate coverslides. Each experiment was repeated at three times.

2.4. Immunocytochemistry

For immunocytochemistry, paraformaldehyde fixed DA neurons were permeabilized with PBS, 0.2% Triton, 1% normal goat serum, 5% bovine serum albumin (30 min at RT) and incubated overnight at 4 °C with anti-TH rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) followed by incubation with a biotinylated goat anti-rabbit antibody (Jackson ImmunoResearch) and ABComplex (Jackson ImmunoResearch) and by development with DAB (Collo et al., 2018a). The samples were visualized with an Olympus IX51 microscope (Olympus Italia Srl, Milan, Italy).

2.5. Computer-assisted morphological analysis

Digital images were acquired with an Olympus IX51 microscope connected to an Olympus (Hamburg, Germany) digital camera and a PC. Morphometric measurements were performed by a blinded examiner on digitalized images using Image-Pro Plus software (Media Cybernetics, Bethesda, MD). Morphological indicators of structural plasticity of DA (TH^+) neurons were considered: (i) the maximal dendrite length, (ii) the number of primary dendrites and (iii) the soma area (Collo et al., 2018a). Maximal dendrite length was defined as the distance from the soma (hillock base) to the tip of the longest dendrite for each neuron; dendrites shorter than 20 μm were excluded from the analysis. Primary dendrites were defined as those directly stemming from the soma. Soma area was assessed by measuring the surface (μm^2) included by the external perimeter drawn on the cell membrane of neurons identified by TH^+ staining. Two coverslides per treatment group per experiment replica were examined, so to obtain measurements from at least 50 neurons, Experiment 1 being replicated 3-times and Experiment 2 and 3 being replicated twice each, respectively.

2.6. Statistical analysis

Data were expressed as mean ± standard error of the mean (S.E.M.). Significant differences from control conditions were determined using Two-Way Analysis of Variance (ANOVA) followed by *a posteriori* Bonferroni's test for multiple comparisons provided by GraphPad Prism, version 9.0 software package (GraphPad Software, San Diego, CA).

3. Results

3.1. Cortisol produces time and dose-dependent impairment of structural neuroplasticity of DA neurons

We studied the effects of cortisol on human DA neurons differentiated from iPSCs at 50 days *in vitro*. At this stage, these neuronal cultures were composed by differentiated and functional DA, glutamatergic and GABAergic neurons (Cavalleri et al., 2018; Collo et al., 2018a). Exposures to cortisol produced hypotrophic effects depending on the different cortisol concentrations and exposure time. Accordingly, a significant main effect of cortisol concentration was observed for maximal dendrite length [$F(3,944) = 27.50$, $p < 0.0001$], number of primary dendrites $F(3,1584) = 16.40$, $p < 0.0001$] and soma area [$F(3,1264) = 14.5$, $p < 0.0001$], the Bonferroni's multiple comparison showing that the most effective concentration was 50 μM ($p < 0.01$) (Fig. 2A–C). A time-dependent main effect was also observed for maximal dendrite length [$F(3,944) = 5.60$, $p < 0.001$] and number of primary dendrites $F(3,1584) = 2.64$, $p < 0.05$], but not soma area [$F(3,1264) = 2.12$, n.s.]. Focusing on the two significant endpoints, for maximal dendrite length multiple comparison revealed significant ($p < 0.001$) effects for 50 μM cortisol at 72h and 96h exposure time vs. vehicle, respectively, while for number of primary dendrites only 50 μM cortisol at 96h showed significant difference ($p < 0.001$). Overall, the most effective condition to affect human DA neuronal structures was the cortisol exposure with the highest concentration and the longest time, i.e., 50 μM/day for 4 consecutive days. Thee data are in keeping with the effects of cortisol-equivalent concentration range described in other hiPSC-derived cell cultures/organoids (Heard et al., 2021; Cruceanu et al., 2022).

The assessment of possible neurotoxic effects of sub-chronic exposure to glucocorticoids, mostly apoptosis, was studied by assessing cell counts and nucleus morphology using DAPI nuclear staining. No difference in DA neuron counts was observed with the regimen of 10 μM and 50 μM daily for 4 days in 3 different experiments vs. vehicle (vehicle = 625.7 ± 3.7, cortisol 10 μM/day = 604.3 ± 3.3, cortisol 50 μM/day = 603.3 ± 3.5). This result suggested that the cortisol concentrations used in the experiment were attenuating neuroplasticity in DA neurons without driving apoptotic cell death, as recently described with similar concentrations in experiments on hiPSC-derived brain organoids (Cruceanu et al., 2022).

3.2. Low doses of ketamine revert the structural deficit of DA neurons produced by cortisol exposure

In the second experiment 1 hr-exposure to various concentrations of ketamine was able to normalize the morphology of human DA neurons previously exposed to high concentration cortisol for 4 days (Fig. 3). Two-way ANOVA showed a significant main effect of ketamine treatment for maximal dendrite length [$F(4,990) = 43.30$, $p < 0.0001$], number of primary dendrites [$F(4, 990) = 13.40$, $p < 0.0001$] and soma area [$F(4,990) = 13.10$, $p < 0.0001$]. A significant interaction in maximal dendrite length [$F(4,990) = 12.80$, $p < 0.0001$], number of primary dendrites [$F(4,990) = 2.69$, $p < 0.05$] and soma area [$F(4,990) = 5.03$, $p < 0.001$] indicated that the effects of ketamine on cortisol hypotrophic effects were dependent upon certain concentrations. Specific information was obtained using Bonferroni's multiple comparisons (Fig. 3 A, B, C), showing that the hypotrophic effects produced by high cortisol pretreatment were significantly ($p < 0.01$-0.0001) rescued by (A) ketamine at all concentration tested for maximal dendrite length, (B) ketamine at concentration >0.05 μM for the primary dendrite number and (C) ketamine at all concentration tested for soma area (Fig. 3). When vehicle was used instead of corticol during the pre-treatment experimental phase, ketamine showed significant neuroplasticity at only at 1 μM dose, lower doses being ineffective, thus confirming previously published data (Cavalleri et al., 2018; Collo et al.,

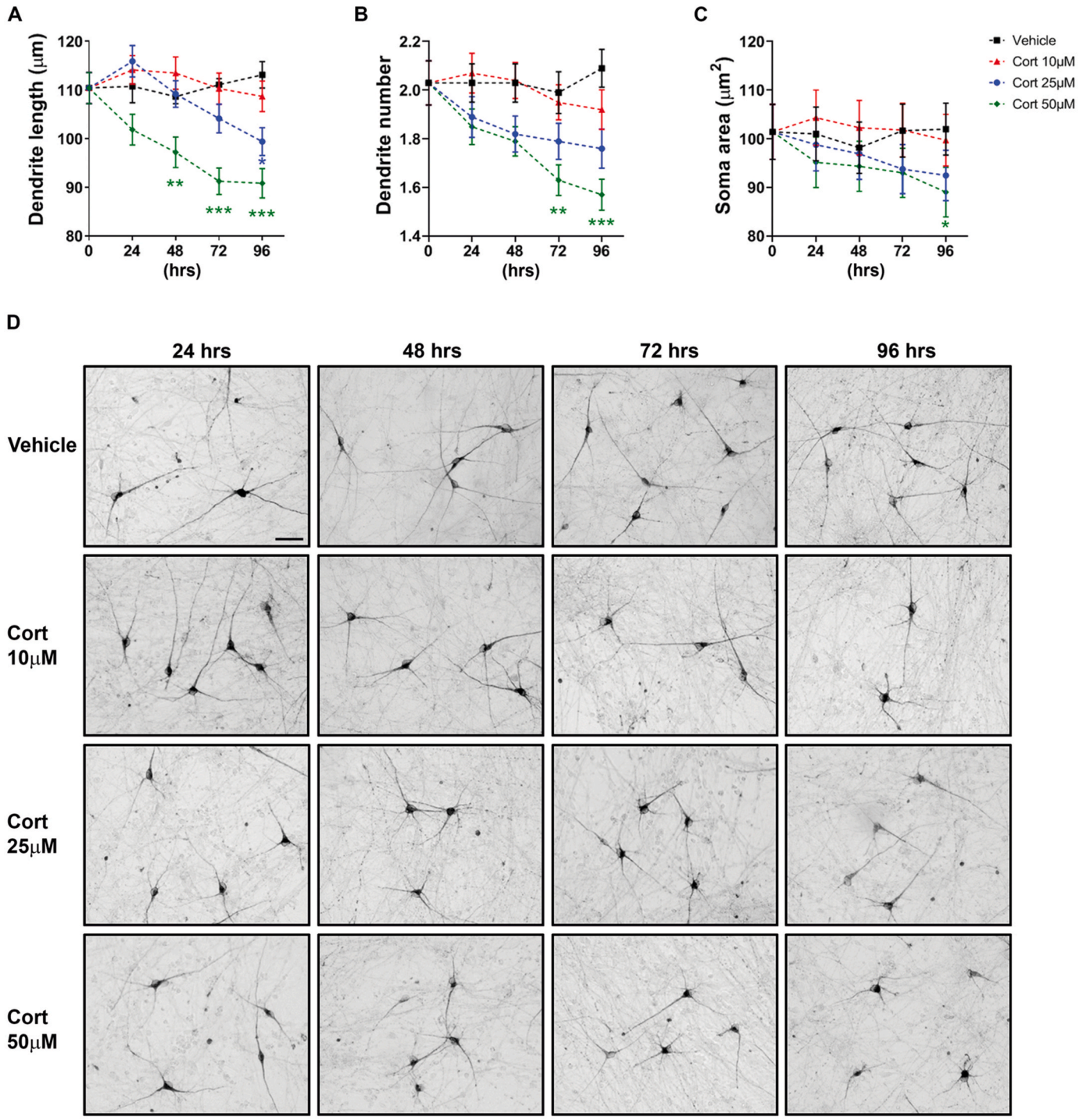

Fig. 2. Concentration- and time-response curve of cortisol effects on structural plasticity. Morphometric quantification of **(A)** maximal dendrite length, **(B)** primary dendrite number and **(C)** soma area performed on TH^+ DA neurons exposed to various concentrations of cortisol (10, 25, 50 µM) or vehicle for various exposure periods (0, 24, 48, 72, 96 h). Data are represented as mean ± SEM (***P < 0.001; **P < 0.01; *P < 0.05 vs. vehicle; post-hoc Bonferroni's test). (**D**) Representative photomicrographs showing the morphological changes of human DA neurons exposed to vehicle and various concentrations of cortisol. Cultures were fixed 72 h after the last treatment and stained with an anti-TH antibody. Scale bar: 50 µm. Cort: cortisol.

2018a).

3.3. Low doses of HNK revert the structural deficit of DA neurons produced by cortisol exposure

The third experiment showed a significant effect of 1h-treatment with HNK in normalizing the morphology of human DA neurons exposed to a pretreatment with high-concentration cortisol for 4 days (Fig. 4). Two-way ANOVA showed a significant main effect of HNK for maximal dendrite length [$F(3,792) = 39.60$, $p < 0.0001$], number of primary dendrites [$F(3,792) = 15.60$, $p < 0.0001$], and soma area [$F(3,792) = 12.10$, $p < 0.0001$], pointing to an overall strong effect. The interaction were also significant for maximal dendrite length [$F(3,792) = 17.10$, $p < 0.0001$], number of primary dendrites [$F(3,792) = 3.26$, p

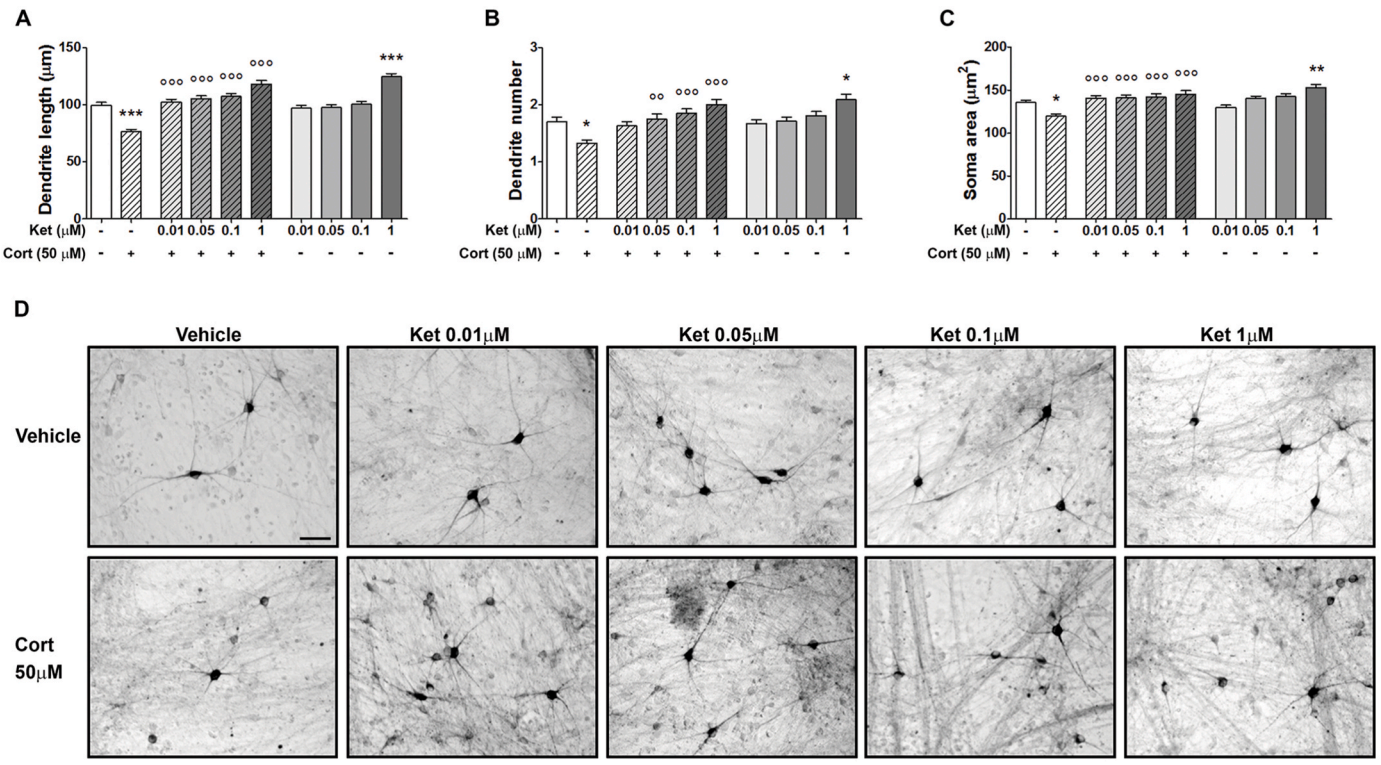

Fig. 3. Low doses of ketamine revert the structural deficit produced by cortisol. Morphometric quantification of **(A)** maximal dendrite length, **(B)** primary dendrite number and **(C)** soma area performed on TH^+ DA neurons previously exposed to cortisol (50 μM) or vehicle for 4 days, followed by exposure to various doses of ketamine (0.01, 0.05, 0.1, 1 μM) or vehicle. Data are represented as mean ± SEM (***P < 0.001; **P < 0.01; *P < 0.05 vs. vehicle; (^{OOO}P<0.001; ^{OO}P<0.01; vs. cortisol; post-hoc Bonferroni's test). (**D**) Representative photomicrographs showing the morphological changes of human DA neurons exposed to vehicle, cortisol (50 μM for 72 h), and vehicle or cortisol followed by ketamine (0.01, 0.05, 0.1, 1 μM) for 60 min. Cultures were fixed 72 h after the last treatment and stained with an anti-TH antibody. Scale bar: 50 μm. Cort: cortisol, Ket: ketamine.

< 0.05], but not for soma area. Multiple comparison (Fig. 4A, B, C) revealed that HNK was effective (p < 0.05-0.0001) on all the morphological endpoints at the low concentrations of 0.05 μM only on DA neurons pretreated with cortisol, while the highest dose of 0.5 μM HNK was effective in both treatment groups. The effect of the latter dose 0.5 μM of HNK in vehicle-exposed human DA neurons was in line to what previously reported (Cavalleri et al., 2018; Collo et al., 2018a).

4. Discussion

In the present exploratory study we used hiPSC-derived DA neurons to model *in vitro* the effects of glucocorticoids on neuronal structural plasticity observed *in vivo*, whose impairment is considered a critical mechanism underlying the symptoms of low mood and anhedonia in MDD/TRD and chronic stress.

We showed that exposures to high-concentrations cortisol (10–50 μM) caused dose-dependent hypotrophic effects on dendrite arborization and soma size of human DA neurons when compared to unexposed control neurons. Recent studies indicated that a reduced length and complexity of dendritic arborization of mesencephalic DA neurons is associated to a lower regularity of their firing rate (Montero et al., 2021), implicating a possible functional role of these cortisol-dependent morphological observations.

It is well-established that different glucocorticoid 'concentration x time' exposures produce different effects in various biological systems (Krugers et al., 2010; Liston and Gan 2011; Bassil et al., 2023). Intriguingly, recent experiments on hiPSC-derived brain cells/organoids indicated that relatively high concentrations of corticol-equivalent exposure (2.5–50 μM) were required to produce gene expression and biochemical changes (Heard et al., 2021; Cruceanu et al., 2022; Bassil et al., 2023), Interestingly, no effect on their morphology was investigated yeat.

It is agreed that relatively high doses of exogenous glucocorticoids (e.g., 10–25 mg/kg/day for weeks) are required to produced results similar to those produced by stress exposure. Stress and high doses glucocorticoids reduced apical dendritic spines and their plasticity in the hippocampus and cortex of rodents (Watanabe et al., 1992, Izquierdo et al., 2006, McEwen and Gianaros, 2011, Liston and Gan 2011, Gold et al., 2015, Anderson et al., 2016, Yau et al., 2016, Barfield and Gourley, 2018). By using dual photon microscopy, Liston and Gan (2011) showed that exposure to moderate/high levels of glucocorticoids for 10 days produced hypotrophic effects achieved through a higher rate of elimination of stable dendritic spines in the primary somatosensory cortex of mice, while low concentration of glucocorticoids for a shorter exposure time displayed the opposite behavior. Moreover, exposure to foot-shock stress strongly increased circulating glucocorticoids and produced dendritic retraction in cortical pyramidal neurons, paralleled by decrease of cofilin phosphorylation (Nava et al., 2017). Intriguingly, these acute stress-induced dendritic retraction was reversible, normalizing spontaneously after several weeks/months (Radley et al., 2005). Conversely, exposures to high-level glucocorticoids for weeks/months, as described in low-rank monkeys under high social stress or in women with severe intractable depression, was associated to reduced dendritic arborization, while apoptosis and atrophy of sensitive neurons of the hippocampus, initially described, as a glutamate-dependent excitotoxicity (Armanini et al., 1990; Popoli et al., 2011), was not always observed (reviewed in Bassil et al., 2023), pointing to a prominent effect via defective neuroplasticity. Acccordingly, the effects on glutamatergic neurotransmission was also present at transient moderate/high concentrations glucocorticoids compatible with a regular stress response (Krugers et al., 2010; Liston and Gan 2011; McEwen and Gianaros, 2011). In a series of studies on various neuronal substrates,

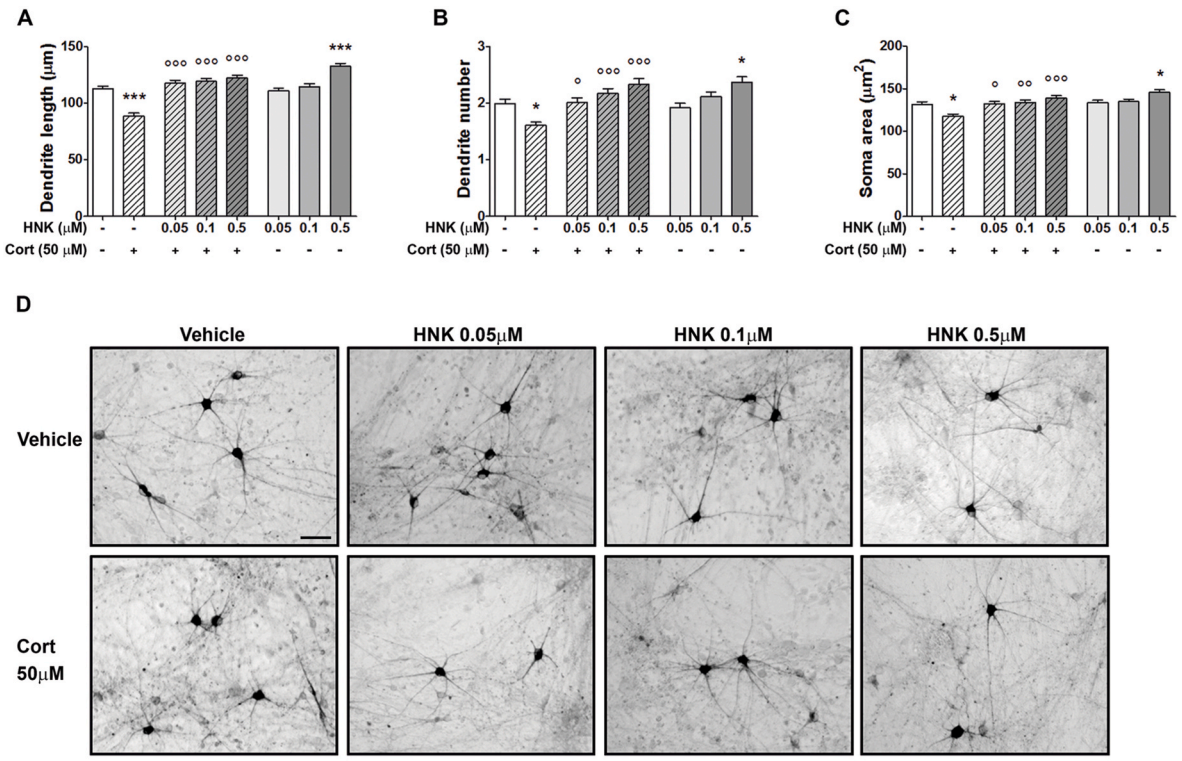

Fig. 4. Low doses of HNK revert the structural deficit produced by cortisol. Morphometric quantification of **(A)** maximal dendrite length, **(B)** primary dentrite number and **(C)** soma area performed on TH^+ DA neurons previously exposed to cortisol (50 µM) or vehicle for 4 days, followed by exposure to various doses of HKN (0.05, 0.1, 0.5 µM) or vehicle. Data are represented as mean ± SEM (***P < 0.001; *P < 0.05 vs. vehicle (^{OOO}P<0.001; ^{OO}P<0.01; ^{O}P < 0.05 vs. cortisol; post-hoc Bonferroni's test); (**D**) Representative photomicrographs showing the morphological changes of human DA neurons exposed to vehicle, cortisol (50 µM for 72 h), and vehicle or cortisol followed by HNK (0.05, 0.1, 0.5 µM) for 60 min. Cultures were fixed 72 h after the last treatment and stained with an anti-TH antibody. Scale bar: 50 µm. Cort: cortisol, Ket: ketamine, HNK: (2R,6R)-hydroxynorketamine.

glucocorticoids were seen to increase the presynaptic level of glutamate and its release in the prefrontal cortex of rodents (Popoli et al., 2011), as well as the membrane levels of AMPAR and NMDAR subunits, changing the sensitivity to glutamatergic input in target neurons (Groc et al., 2008; Krugers et al., 2010; Tse et al., 2012; Popoli et al., 2011).

In the present study, four-day exposures to high levels of cortisol produced a marked increase in the sensitivity of the structural plasticity response to the glutamatergic agents ketamine and HNK in human DA neurons differentiated from iPSCs. These two agents consistently triggered a significant dendritic outgrowth and soma size increase in human DA neurons previously exposed to moderate/high subacute doses of hydrocortisone. These structural neuroplasticity events were produced at concentrations approximately 10- or 100-fold lower than those necessary to trigger the same response in vehicle-exposed DA neurons. In a previous study using the same iPSC-derived human DA neuron platform, we showed that the effects of ketamine and HNK were blocked by pretreatment with the AMPA receptor blockers NQBX and GYKI 52466 (Cavalleri et al., 2018). Intriguingly, similar AMPAR-mediated effects on dendritic spines of hippocampal and cortical neurons were observed in rodents after chronic stress, where neuroplasticity effects were also reverted by ketamine (Li et al., 2010). Ketamine effects on iPSC-derived human DA neurons were observed at 1.0 µM (Cavalleri et al., 2018), while in the present study, in iPSC-derived human DA neurons exposed to cortisol for 4 days, ketamine was effective in the range of 0.01–1.00 µM. These concentrations were in the range of the exposures (0.20–0.80 µM) measured in a pharmacokinetic study in TRD patients that responded to ketamine treatment (Zhao et al., 2012). Similar dose-related efficacious exposures were observed in other studies in MDD/TRD patients (Zarate et al., 2006; Diazgranados et al., 2010; Serafini et al., 2014). Intriguingly, the observed sensitization produced by cortisol treatments in iPSC-derived human DA neurons extended also to HNK, one of the main metabolites of ketamine, currently considered a new putative glutamatergic antidepressant (Zanos et al., 2016). In fact, HKN showed effects at exposures 10-times lower that those observed in human DA neurons not previously exposed to cortisol (i.e., 0.05 µM vs. 0.50, µM, respectively). Intriguingly, these lower concentrations were in the range (0.05–0.10 µM) to those observed in patients in the human pharmacokinetic study of Zhao et al. (2012) that profiled ketamine and its metabolites, including HNK, in TRD subjects whose depression score improved significantly.

It is tempting to speculate that the limbic neuroplasticity impairment associated to the lack of response to standard SSRI antidepressant treatment in TRD could be related to the presence of an excessive glucocorticoid level, often not measured in the protocol, requiring a therapy with ketamine. However, so far, only a weak correlation between glucocorticoid levels and lack of response to treatment was described (Perrin et al., 2019; Nikkheslat et al., 2020), suggesting the presence of other predisposing factors in TRD patients. For example, a correlation between childhood trauma and glucocorticoid resistance was described (Nikkheslat et al., 2020), with the implication that the childhood trauma experience may generate a long-standing epigenetic predisposition that would be further enhanced by high glucocorticoids, contributing to the TRD condition. This observation is in keeping with the evidence of serious mental illness triggered by the therapeutic use of high doses of synthetic glucocorticoids prescribed for anti-inflammatory purposes. Accordingly, in a large epidemiological study, exposure to synthetic glucocorticoids showed a 7-fold higher probability of suicide and 2-fold higher occurrence of MDD when compared to the normal population (Fardet et al., 2012). These data also suggest the presence of predisposing factors in certain individuals, whose neurons could be more susceptible to glucocorticoid-mediated impairments of neuronal plasticity.

This exploratory study has some intrinsic limitations in view of the recently published recommendations on iPSC methodology for pharmacological studies in neuropsychiatric disorders (Polit et al., 2023). In fact, it was run using only one highly reliable iPSC line from a healthy donor successfully used in several studies, including Cavalleri et al. (2018). Another limitation is the lack of functional tests showing the engagement of specific cortisol-dependent molecular mechanisms, as well as a longitudinal follow-up study showing a possible recovery after high cortisol exposure.

In conclusion, in this exploratory study we provide the first evidence that the impaired neuronal structural plasticity produced by glucocorticoids is reversed by the rapid-acting antidepressant ketamine at concentrations compatible within the range observed in patients. The similar response observed with ketamine and HNK in this *in vitro* hypercortisolism model could eventually contribute to support the rationale for progressing HNK development as new MDD/TRD therapeutic.

Credit author statement

Conceptualization: GC, EMP, MZ.
Methodology: GC, EMP.
Formal analysis: LC, GC, EMP.
Investigation: LC, ID, GSM.
Writing – original draft: GC, EMP.
Writing – review and editing: GC, EMP, MZ.
Supervision: GC.
Funding acquisition: GC.

Author disclosure

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Data availability

The data underlying this article are available in the article. Request for the value of each individual data point will be provided upon reasonable request to the corresponding author.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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