HELLENIC REPUBLIC

National and Kapodistrian University of Athens

Department of Biology

Athens International Master's Programme in Neurosciences

Biomedical Research Foundation Academy of Athens

# RESEARCH THESIS PROJECT

Modulators of alpha-synuclein-mediated neurodegeneration: Evaluation of an in vitro stress modality on alpha-synuclein transcriptional regulation, expression, accumulation, and aggregation in cultured primary hippocampal neurons derived from wild-type and humanized alpha-synuclein rats.

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2018

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2018

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**Τροποποιητές του διαμεσολαβούμενου από την άλφα-συνουκλεΐνη νευροεκφυλισμού:** Αξιολόγηση μιας in vitro μεθόδου καταπόνησης στη μεταγραφική ρύθμιση, έκφραση, συσσώρευση και συσσωμάτωση της άλφα-συνουκλεΐνης σε καλλιεργημένους πρωτογενείς νευρώνες του ιπποκάμπου προερχόμενους από αρουραίους άγριου-τύπου και εξανθρωπισμένους α-συνουκλεϊνικούς αρουραίους.

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## Περίληψη

Το χρόνιο άγχος έχει προταθεί ως ένας από τους περιβαλλοντικούς παράγοντες που προκαλούν τη νόσο του Πάρκινσον. Η υπερέκφραση αλφα-συνουκλεΐνης συνδέεται γενετικά με τη νόσο του Πάρκινσον αλλά και παθολογικά καθώς τοξικές μορφές της πρωτεΐνης και τα συσσωματώματα της είναι παρόντα σε εγκεφάλους ασθενών μετά θάνατον. Εδώ, δείχνουμε ότι το συνθετικό γλυκοκορτικοειδές, η δεξαμεθαζόνη, έχει επιπτώσεις στη μεταγραφή, την έκφραση και την έκκριση της α-συνουκλεΐνης σε άγριου τύπου και σε προερχόμενες από διαγονιδιακούς αρουραίους που υπερεκφράζουν την ανθρώπινη άλφα-συνουκλεΐνη διαγονιδιακές πρωτογενείς νευρωνικές καλλιέργειες ιπποκάμπου και φλοιού. Η θεραπεία με δεξαμεθαζόνη αυηάνει τα επίπεδα mRNA του SNCA (γονιδίου της αλφα-συνουκλείνης) αρουραίου και ανθρώπου τόσο σε ιπποκάμπου όσο και σε φλοιού πρωτογενείς νευρωνικέ καλλιέργειες. Αυτή η ρύθμιση που καθοδηγείται από τη δεξαμεθαζόνη μεταφράζεται σε υπερέκφραση αλφα-συνουκλεϊνης και υψηλότερα επίπεδα φωσφορυλιωμένης (pSER-129) άλφα-συνουκλεΐνης κυρίως σε πρωτογενείς νευρωνικές καλλιέργειες ιπποκάμπου. Υπάρχει απουσία εξαρτώμενης από τη δεξαμεθαζόνη έκκριση α-συνουκλεϊνης τόσο σε πρωτογενή θρεπτικά μέσα καλλιέργειας ιπποκάμπου όσο και στα μέσα καλλιέργειας φλοιού. Σε διαγονιδιακές συνθήκες καλλιέργειας, τα επίπεδα πρωτεΐνης της εκκρινόμενης α-συνουκλεϊνης στο θρεπτικό μέσο καλλιέργειας είναι υψηλότερα, ακόμη και απουσία δεξαμεθαζόνης, σε σύγκριση με τα μέσα καλλιέργειας των αγρίου τύπου νευρωνικών καλλιεργειών τόσο στον ιππόκαμπο όσο και στον φλοιό.

**Λέξεις-Κλειδιά:** στρες, άλφα-συνουκλεϊνη, δεξαμεθαζόνη, ασθένεια του Πάρκινσον, μεταγραφική ρύθμιση, έκκριση, πρωτογενείς νευρωνικές καλλιέργειες ιπποκάμπου και φλοιού, εξανθρωπισμένοι άλφα-συνουκλεϊνικοί αρουραίοι

# Modulators of alpha-synuclein-mediated neurodegeneration: Evaluation of an in vitro stress modality on alpha-synuclein transcriptional regulation, expression, accumulation, and aggregation in cultured primary hippocampal neurons derived from wild-type and humanized alpha-synuclein rats.

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## Highlights

- Dexamethasone seems to increase rat and human SNCA mRNA in hippocampal and cortical primary neuronal cultures derived from human alpha-synuclein overexpressing BAC (hu-AS BAC) rats
- Dexamethasone treatment has differential effects on alpha-synuclein protein levels and its phosphorylation (ser129) in wildtype and hu-AS BAC hippocampal and cortical primary neuronal cultures
- hu-AS BAC hippocampal and cortical primary neuronal cultures secrete higher levels of alpha-synuclein compared to wildtype
- Endogenous total alpha-synuclein levels are higher compared to secreted alpha-synuclein levels in hu-AS BAC hippocampal neuronal cultures

## Summary

Chronic stress has been proposed as one of the environmental triggers of Parkinson's disease. Alpha-synuclein overexpression is linked genetically to Parkinson's disease but also pathologically as toxic forms of the protein and aggregates are present in post mortem patient brains. Here, we show that the synthetic glucocorticoid, dexamethasone, has effects on transcription, expression and secretion of a-synuclein in wildtype and BAC human alpha-synuclein overexpressing-transgenic primary hippocampal and cortical neuronal cultures. Dexamethasone treatment up-regulates rat and human SNCA mRNA both in hippocampal and cortical primary neuronal cultures. This dexamethasone-driven up regulation translates into a-synuclein overexpression and higher levels of phosphorylated (pSER-129) alpha-synuclein primarily in primary hippocampal neuronal cultures. There is an absence of dexamethasone-dose dependent secretion of a-synuclein both in primary hippocampal and cortical cultured condition media. In BAC-transgenic cultured condition media secreted a-synuclein protein levels are higher, even in baseline conditions, compared to WT cultured condition media both in hippocampus and cortex.

## Keywords

• stress • alpha-synuclein • dexamethasone • Parkinson's disease • transcriptional regulation

• secretion • primary hippocampal neurons • primary cortical neurons • humanized alpha-synuclein rat

## Introduction

Parkinson's disease (PD) is a late-onset neurodegenerative disorder which represents a growing health concern for an ever-aging population. The cardinal manifestations of PD include resting tremor, bradykinesia (slowed movements), rigidity (increased muscular tone), postural instability, and gait impairment (Fahn S. 2003). The neuropathological hallmarks of PD are selective depletion of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and the formation of Lewy body (LB) aggregates (Shulman JM, De Jager PL, Feany MB., 2011). The main component of Lewy bodies is the presynaptic protein α-synuclein (AS), aggregation of which is thought to be pathogenic in a family of diseases termed synucleinopathies, which includes PD, multiple system atrophy and Lewy body Dementia (DLB) (Brettschneider et al., 2015; Luk et al., 2012; Prusiner et al., 2015). Impaired motor function is a classic sign used in the clinical diagnosis of PD, however, those affected with PD also harbor non-motor symptoms including olfactory deficits, sleep disturbances, reduced stress tolerance, depression and cognitive decline, which may precede the onset of motor symptoms more than 20 years (Chaudhuri and Schapira, 2009). While genetic risks have been identified, environmental factors and gene-environment interactions likely account for most PD cases (Nalls et al., 2014), as less than 10% of cases are hereditary (Nalls et al., 2014).

In parallel with the growing appreciation that, clinically, PD causes a host of non-motor manifestations, it has been recognized that α-synuclein pathology ranges beyond the SNpc into much of the neuraxis (Burke, EB, Dauer W.T, Vonsattel JPG, 2008). Although it is now widely acknowledged that Lewy pathology can be found throughout the nervous system, the Braak staging hypothesis of a stereotyped caudal-to-rostral spread of pathology remains an attractive hypothesis waiting for a definitive confirmation. Accordingly, the investigation of PD pathological mechanisms, including the development of animal models, has focused largely on the vulnerability of dopaminergic cells in the SN. However, there remains an unmet need to understand how this disease spreads beyond the basal ganglia to other brain systems.

Can chronic stress cause or exacerbate Parkinson's disease? Stress has been one of the earliest proposed causes of PD (Charcot, 1872; Gowers, 1888). Several studies have suggested that acute or chronic stress might lead to earlier onset or worsen the motor symptoms of PD (Gibberd & Simmons, 1980; Weiner & Lang, 1989; Goetz et al., 1990; Treves *et al*., 1990; Smith et al., 2002). Brain regions participating in motor

control, such as the motor cortex, cerebellum and the basal ganglia, show considerable density of glucocorticoid receptors (Ahima & Harlan, 1990; Ahima et al., 1991), rendering these areas susceptible to the effects of stress. Accordingly, stress and stress hormones were shown to affect the function of the intact motor system in both human (Maki & McIlroy, 1995) and rat (Metz et al., 2001a, 2005). Additionally, one study indicated that cortisol levels are positively associated with gait deficits in Parkinson patients (Charlett et al., 1998). In accordance with studies showing that the dopaminergic system is particularly susceptible to the effects of stress (Finlay & Zigmond, 1997; Pani *et al*., 2000; Izzo et al., 2005), it has been demonstrated that oxidative stress caused by immobilization stress selectively damages the nigrostriatal dopaminergic system (Kim et al., 2005).

The glucocorticoid receptor and mineralocorticoid receptor mediate the cellular response to corticosteroids secreted from the adrenal glands. These adrenal steroid receptors are present in various brain regions, exhibiting their highest expression in the hypothalamus and hippocampus, as it was observed both in humans and rodents (Ahima 1991, Wang 2013). Utilizing genomic and non-genomic mechanisms, glucocorticoid receptors and mineralocorticoid receptors can modulate multiple neurochemical systems, including the primary neurotransmitters glutamate and GABA; the monamine neurotransmitters serotonin, (5-hydroxytryptamine, 5-HT), noradrenaline and dopamine; neurotrophic factors, such as brain-derived neurotrophic factor (BDNF); and other extracellular signaling systems, such as endocannabinoids (Alangari A.A., 2010; GB Makara, J. Haller 2001; Micale V and Drago F , 2018). Repeated or chronic exposure to stressors, however, might deleteriously affect this exquisitely tuned system. In rodent models, chronic stress is associated with dendritic remodelling, impaired learning and memory, and increased anxiety and depressive-like behaviours (Watanabe 1992, Conrad 1996). In addition, stress accelerates neural degeneration and exacerbates motor symptoms in a rat model of Parkinson's disease (Smith LK et al., 2008). In humans, chronic stress is a major risk factor for accelerating the onset of and exacerbating symptomatology of mental health disorders such as depression and anxiety-both of which have significant comorbidity (22 and 25-40%, respectively) as non-motor symptoms of PD (Pfeiffer et al., 2016). A recent study of Park et al., 2013, showed that dexamethasone, a synthetic glucocorticoid, induces the expression of LRRK2 and a-synuclein, two genes that when mutated cause Parkinson’s disease in an autosomal dominant manner, in dopaminergic cell lines and in primary rat hippocampal neurons. In addition, glucocorticoid receptors (GR) of the microglia also play a pivotal role in regulating dopaminergic neurodegeneration, highlighting the role of GR in the inflammation component of neurodegeneration (Ros-Bernal F. et al., 2011).

Based on the aforementioned data, we hypothesized that chronic stress may lead to exacerbation of alpha-synuclein pathology directly through transcriptional up-regulation of SNCA. Consequently, in our study we examined the mechanisms involved in potential direct effects of a synthetic glucocorticoid, dexamethasone (DEX), on AS gene (SNCA) transcription in cultured hippocampal and cortical neurons of wildtype (WT) and humanized alpha-synuclein rats (BAC). Our results showed that dexamethasone increased both a-synuclein mRNA (rat and human) and protein levels of WT and BAC cultured primary hippocampal and cortical neurons. In addition, we identified a DEX-dependent increase in AS phosphorylation at the ser129 residue a post-translational modification that is widely thought to be linked to PD pathology (Smith et al., 2005, Fujiwara et al., 2002). Finally, we were able to detect protein levels of secreted AS in cultured condition media of our neuronal culture models and to identify a DEX-dependent increase of secreted AS in WT and to a greater extent in BAC hippocampal and cortical neurons. Under physiological conditions, the role of glucocorticoids is debatable and probably depends on the brain region, GR expression levels and DEX dosing. Our results suggest that stress activation alone can alter the transcriptional regulation of AS as demonstrated in WT hippocampal and cortical neurons. In addition, we demonstrate for the first time that in primary hippocampal and cortical neurons that overexpresses human AS, stress system activation leads to the induction of transcriptional and translational regulation of AS. The combination of stress system activation and increased AS burden models the "double hit" hypothesis of non-familial PD pathogenesis, thus providing a model for the gene-environment causality of PD.

# Methods

## EXPERIMENTAL MODEL AND SUBJECT DETAILS

### RAT MODEL

We utilized a bacterial artificial chromosome (BAC) α-synuclein transgenic rat model carrying the full-length human wild-type SNCA locus, including all introns and exons, the upstream localized regulatory promoter sequences and parts of the 3'-untranslated region. This BAC-transgenic model of PD overexpresses human AS (hu-AS) and exhibits increased AS pathology including age-dependent accumulation of proteinase K resistant and insoluble oligomeric forms of AS and C-terminal truncated AS as well as a behavioral phenotype recapitulating some changes seen in early and late Parkinson's disease stages (Nuber et al.,2013).

## Animal Statement

All efforts were made to minimize animal suffering and to reduce the number of the animals used, according to the European Communities Council Directive (86/609/EEC) guidelines for the care and use of laboratory animals. All animal experiments were approved by the Institutional Animal Care and Use Committee of Biomedical Research Foundation of the Academy of Athens.

## METHOD DETAILS

### Primary Neuronal Cultures

Cultures of Sprague Dawley (SD) rat (embryonic day 17) cortical and hippocampal neurons were prepared as previously described (Rideout, H. J., and Stefanis, L., 2002) from wildtype or hu-AS rats. 600.000 cells for cortical cultures or 450.000-550.000 cells for hippocampal cultures were plated onto poly-D-lysine-coated twelve-well dishes. Cells were maintained in Neurobasal medium (Invitrogen, Carlsbad, CA, USA), with B27 serum-free supplement (Invitrogen, Carlsbad, CA, USA) for cortical cultures or N21 medium supplement (50X) (Molteni-Macs neurobrew 21) for hippocampal cultures, L-glutamine (1,5 mM), and penicillin/streptomycin (1%). More than 98% of the cells cultured under these conditions represent post-mitotic neurons (Rideout, H. J., and Stefanis, L., 2002). Cultures were maintained either in 0% or 2.5% fetal bovine serum (FBS) (10,270; Gibco, Invitrogen, Carlsbad, CA, USA), concentration upon treatment at 6 DIV. All detailed primary culture conditions and troubleshooting are summarized in Table 1.

### Western Immunoblotting

Cultured cells of embryonic rat cortical and hippocampal neurons, were washed twice in cold PBS and then lysed using STET lysis buffer (50 mM Tris, pH 7, 6, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA) with protease inhibitors. Lysates left on ice for ~ 30 min and then centrifuged at 10.000xg for 20 min at 4°C and the supernatant containing the Triton-X-soluble proteins was collected. Protein concentrations were determined using the Bradford method (Bio-Rad, Hercules, CA, USA). The pellets were then lysed in RIPA buffer (150 mM NaCl, 50 mM Tris pH7.6, 0.1% sodium dodecyl sulfate, 1% NP-40; Sigma, 2 mM EDTA) with protease inhibitors. Lysates left on ice for ~ 30 min and then centrifuged at 10.000xg for 20 min at 4°C and the supernatant containing the SDS -soluble proteins was collected. Protein concentrations were determined using the DC protein assay from Bio-Rad. All buffers were supplemented with protease (11836170001; Roche, Mannheim, Germany) and phosphatase inhibitors (04406837001; Roche, Mannheim, Germany). Twenty to thirty micrograms of lysates were mixed with 4x Laemmli buffer prior to running on 12% or 13% (for ASYN) SDS–polyacrylamide gels. Following transfer to a nitrocellulose membrane, blots were probed with the following antibodies: total a-synuclein (SYN1, 1:1000, BD

Transduction Laboratories), pSER(129)-a-synuclein (1:1000, Imago Pharmaceuticals), loading control, γ-tubulin (1:2000). Blots were probed with horseradish peroxidase-conjugated secondary antibodies and visualized with enhanced chemiluminescence substrate (ECL) following exposure to Super RX film (FUJI FILM, Europe GmbH, Germany). The intensity of each immunoreactive band was estimated by densitometric quantification using ImageJ software.

### RNA Extraction and cDNA Synthesis

Total RNA was extracted from primary hippocampal and cortical neuronal cultures using TRIzol® (Ambion, Thermo Fisher Scientific, Waltham, MA, USA). Digestion with 1 U/μg DNase I (Promega, Madison,WI, USA), was utilized to remove any remaining DNA. RNA concentration was determined spectrophotometrically at 260 nm, although the quality of purification was determined by a 260:280-nm ratio that showed values between 1.5 and 2.0, indicating high RNA quality. 1 μg total RNA was used for first-strand cDNA synthesis with the Moloney murine leukemia virus reverse transcription system (Promega, Madison, WI,USA) and utilized for real-time PCR, according to the manufacturer's instructions. For the reaction we used 1–2ug of total RNA, 1x buffer, 500 ng of oligo(dT) primer, 2 mM dNTPs, 40 units of RNasin, and 200 units of Moloney murine leukemia virus enzyme.

### Reverse Transcription-PCR

Duplicates of each sample were assayed by relative quantitative real-time PCR using a Light Cycler 96 (Roche Applied Science, Mannheim, Germany) machine to determine the mRNA expression levels of rat *SNCA* and human *SNCA*. As a reference gene for normalization, we used β-actin. The primers used for rat *SNCA* were 5′-GCCTTTCACCCCTCTTGCAT-3′ (forward), 5′-TATCTTTGCTCCACACGGCT-3′ (reverse), for human *SNCA* were 5′-CCGCTCGAGCGGTAGGACCGCTTGTTTTAGAC-3′ (forward), 5′-CCTCTTTCCACGCCACTATC-3′ (reverse) and for β-actin, 5′-TGGCTCCTAGCACCATGA-3′ (forward), 5′-CCACCAATCCACACAGAG-3′ (reverse). Each cDNA sample was diluted 1:20 before use in the amplification assay. The PCR conditions were as follows: 1× buffer (-Mg), 1.5 mm $MgCl_2$, 0.2 mm dNTPs, 0.2 μm primers, template < 500 ng, 2 U Platinum *Taq*, and SYBR Green (Roche, Mannheim, Germany). The PCR cycling conditions were as follows: 95°C for 180 s, 95°C for 10 s, 60°C for 15 s, and 72°C for 15 s (45 cycles), and 95°C for 60 s, 65°C for 60 s, 95°C for 10 s, and 37°C for 30 s. As a negative control for the specificity of amplification, we used no template samples in each plate; no amplification product was detected in the control reactions. Data were analyzed automatically with a threshold set in the linear range of amplification. The cycle number at which any particular sample crossed that threshold (*Ct*) was used to

determine fold difference, whereas the geometric mean of the control gene (β-actin) served as a reference for normalization. Fold difference was calculated with the $2{-}\Delta\Delta Ct$ method.

### Ultra-sensitive ELISA

For the sandwich ELISA, the monoclonal Syn-1 antibody (BD Biosciences), raised against amino acids 15–123 of the human, mouse or rat α-synuclein sequence, was used as capture antibody. This antibody recognizes a conserved epitope in human and rodent α-synuclein (residues 91–99) whereas it shows no reactivity for the β- or γ-synuclein isoforms. The polyclonal C-20 antibody (Santa Cruz), raised against a C-terminus peptide of human α-synuclein, was used for antigen detection through direct conjugation with HRP (Pierce). Each ELISA plate (Corning Costar) was coated for 24 hrs at room temperature with 0.5 μg/ml of Syn-1 (50 μl per well) in 100 mM $NaHCO_3$, pH 9.3. The plates were washed three times in wash buffer (50 mM Tris-HCl, 150 mM NaCl and 0.04% Tween-20) and 50 μl of protein cell lysate (Triton-X soluble) or cultured condition media and recombinant α-synuclein (as standard), appropriately diluted in TBST/BSA (10 mM Tris-Cl, pH 7.6, 100 mM NaCl, 0.1% Tween-20 and 1% BSA) was added. To allow antigen binding, plates were incubated at 37°C for 2 ½ hrs. After washing three times with wash buffer, 50 μl of HRP-conjugated C-20 antibody (2500x diluted in TBST/BSA) were added to each well and further incubated for 1 hr at ambient temperature. The wells were washed and 50 μl of chemiluminogenic HRP substrate (ultrasensitive luminol reagent, BioFX Laboratories) were added to each well. The wells were incubated for 15 min at room temperature and the chemiluminescence was integrated for 1 s (Emmanouilidou et al., 2011).

## QUANTIFICATION AND STATISTICAL ANALYSIS

### Statistics

No statistical methods were used to predetermine sample sizes. Data presented as bar graphs indicate mean ± standard error of the mean (SEM). Statistical analysis was performed using the unpaired two-tailed Student's t-test, for single comparisons. Where multiple testing was required, one-way ANOVA (treatment) or two-way ANOVA tests (treatment x genotype) were utilized. Statistical details of experiments can be found in the figure legends or main text. Significance levels are indicated as follows: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$. All statistical analyses were performed using the GraphPad Prism 6 suite of software (GraphPad Software, Inc., San Diego, CA, USA).

| genotype | Primary Neuronal culture | RAT species | medium conditions | FBS (%) | DEX dose (uM) | Day of treat-ment | Day of RNA extraction | Day of protein isolation | Culture comments |
|---|---|---|---|---|---|---|---|---|---|
| WT | cortical | Wistar | NB,B27 | 0 | 0.1,1,10 | DIV5 | DIV6 | DIV7 | Stressed after treatment |
| WT | cortical | SD | NB,B27 | 0 | 1, 5, 10 | DIV6 | DIV7 | DIV8 | Stressed after treatment |
| WT | cortical | SD | NB,B27 | 0 | 1, 5, 10 | DIV6 | DIV7 | DIV8 | Stressed after treatment |
| BAC | cortical | SD | NB,B27 | 0 | 1, 5, 10 | DIV6 | DIV7 | DIV8 | Stressed after treatment |
| WT | cortical | SD | NB,B27 | 0 | 1, 5, 10 | DIV6 | DIV7 | DIV8 | good |
| BAC | cortical | SD | NB,B27 | 0 | 1, 5, 10 | DIV6 | DIV7 | DIV8 | good |
| BAC | cortical | SD | NB,B27 | 10 | 1, 5, 10 | DIV6 | DIV7 | DIV8 | good/ high glia content |
| WT | cortical | SD | NB,B27 | 10 | 1, 5, 10 | DIV6 | DIV7 | DIV8 | good/ high glia content |
| BAC | cortical | SD | NB,B27 | 2.5 | 1, 5, 10 | DIV6 | DIV7 | DIV8 | good |
| WT | cortical | SD | NB,B27 | 2.5 | 1, 5, 10 | DIV6 | DIV7 | DIV8 | good |
| WT | cortical | SD | NB,B27 | 2.5 | 0.1,1,5, 10 | DIV6 | DIV9 | DIV9 | good |
| BAC | cortical | SD | NB,B27 | 2.5 | 0.1,1,5, 10 | DIV6 | DIV9 | DIV9 | good |
| WT | hippoca mpal | SD | NB,N21 | 10 | 1, 5, 10 | DIV6 | DIV7 | - | good/ high glia content |
| WT | hippoca mpal | SD | NB,N21 | 2.5 | 1, 5, 10 | DIV6 | DIV7 | - | good |
| WT | hippoca mpal | SD | NB,N21 | 2.5 | 1, 5, 10 | DIV6 | DIV7 | - | good |
| BAC | hippoca mpal | SD | NB,N21 | 2.5 | 1, 5, 10 | DIV6 | DIV7 | - | good |
| BAC | hippoca mpal | SD | NB,N21 | 2.5 | 1, 5, 10 | DIV6 | DIV7 | - | good |
| BAC | hippoca mpal | SD | NB,N21 | 2.5 | 1, 5, 10 | DIV6 | - | DIV8 | good |
| BAC | hippoca mpal | SD | NB,N21 | 2.5 | 1, 5, 10 | DIV6 | - | DIV8 | good |
| BAC | hippoca mpal | SD | NB,N21 | 2.5 | 1, 5, 10 | DIV6 | - | DIV8 | good |
| WT | hippoca mpal | SD | NB,N21 | 2.5 | 1, 5, 10 | DIV6 | - | DIV8 | good |
| WT | hippoca mpal | SD | NB,N21 | 2.5 | 1, 5, 10 | DIV6 | - | DIV8 | good |

**Table 1. Primary Neuronal Cultures conditions and troubleshooting.**

The first primary cortical cultures were maintained in 0% FBS Neurobasal Medium with B27 supplement at 3 different host labs. Due to problems in our lab that made it impossible in the previous year to have viable primary neuronal cultures and after certified cleaning and sterilization procedures, we proceeded with extensive troubleshooting of primary cultures and we finally succeeded in maintaining viable primary neuronal cultures 6 months into the project. The conditions we used finally were 10% FBS enriched Neurobasal 0 DIV that was gradually diminished to 2.5% FBS at 6 DIV (day of treatment). We chose the gradual decrease in FBS concentration rather than adding antimitotic drugs because when we did, we had

100% cell death. B27 supplement was chosen for primary cortical cultures and N21 for hippocampal cortical cultures. With these conditions, we had the best neuronal network production, the minimum cell death and glia content. Primary cultures that were entirely sustained in 10% FBS were excluded from the results of this paper, since the high content of glial cells could interfere with the results. In the case of primary cortical cultures, since the data obtained from 0 and 2.5% FBS enriched media had the same trends, samples were pooled.

## Results

### Dexamethasone Induces Expression of Human and Rat SNCA in Both Primary Hippocampal and Cortical Neurons

Overexpression of WT AS by gene duplication or triplication causes familial PD (Singleton et al., 2003; Farrer et al., 2004). In addition, AS is a major component of the Lewy body, a pathological marker of PD. Here, we utilize a BAC-transgenic model of PD that overexpresses human AS (hu-AS) and exhibits increased AS pathology including age-dependent accumulation of proteinase K resistant and insoluble oligomeric forms of AS and C-terminal truncated AS (Nuber et al. 2013) as well as increased levels of ser129 phosphorylated AS (unpublished observations). Thus, this model is ideal to recapitulate in the in vivo but also in vitro environment the pathological hallmarks of the disease.

The primary goal was to elucidate whether DEX administration regulates SNCA gene transcription in WT and in BAC-transgenic primary neuronal cultures; we thus wanted to test the hypothesis that stress signaling induce cardinal upstream modifications in an environment primed for the development of PD-related pathological events.

The steroid receptors, glucocorticoid and mineralocorticoid receptors, mediate the cellular response of corticosteroids secreted from the adrenal glands, they are present in various brain regions and as recently identified, are most highly expressed in the hypothalamus and hippocampus in both humans and rodents (Ahima et al., 1991 ; Wang et al., 2013). For this reason, the potent synthetic glucocorticoid, dexamethasone, was administered in cultured embryonic rat hippocampal neurons. In addition to the hippocampus, the cortex was also selected for the investigation of direct and indirect effects of dexamethasone. Cortical neurons represent another model cell system appropriate for studying SNCA transcriptional regulation since, as our lab previously reported (Rideout HJ. Et al., 2003), these neurons exhibit a rich, homogeneous source of CNS neurons that mature in culture and during this process, SNCA protein levels are up-regulated. Additionally, cortical neurons are affected at the later stages of PD

progression where the SNCA pathology correlates with the impaired cognition observed in PD with dementia (Mattila et al 2000).

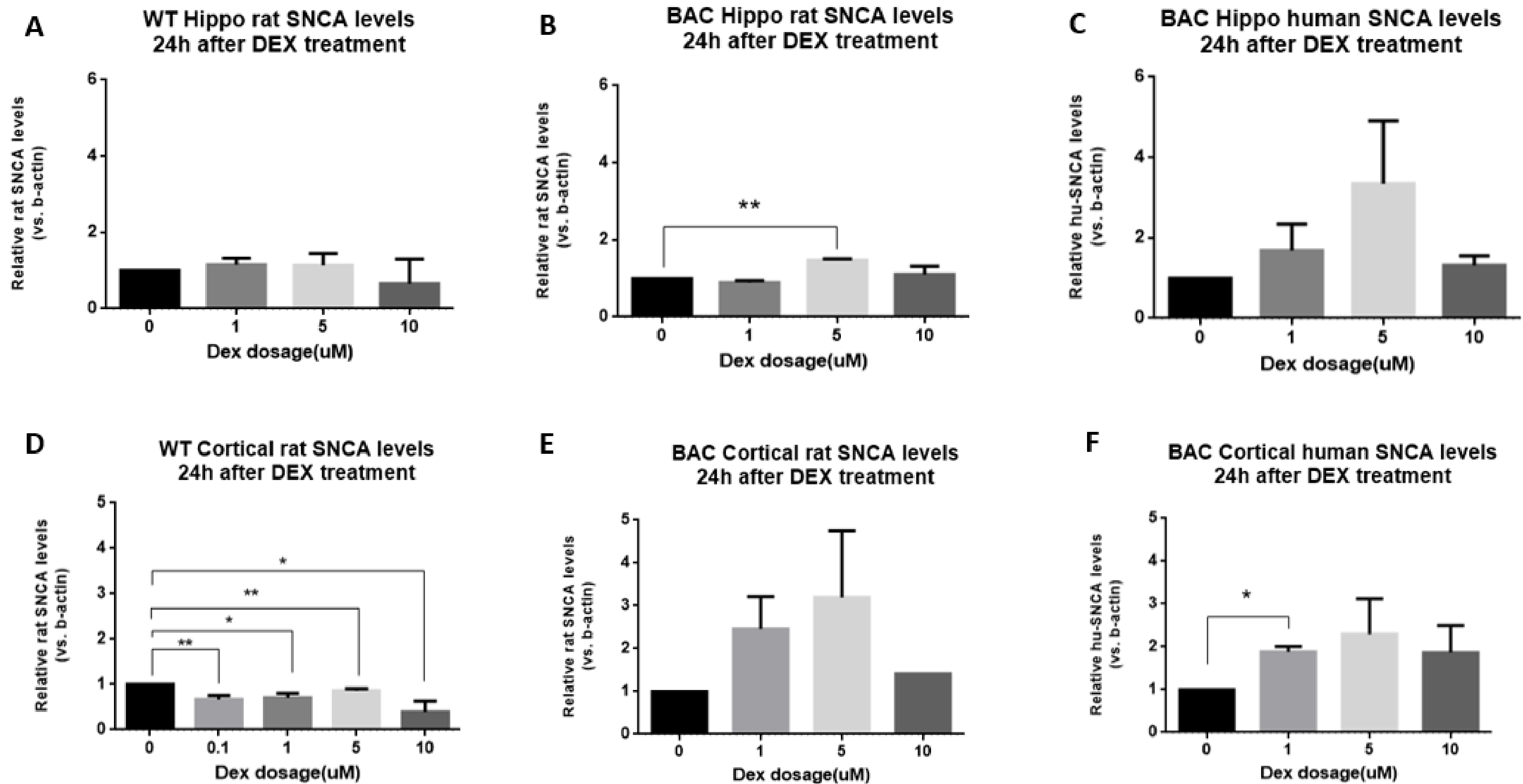


**Figure 1. Dexamethasone Induces Expression of Human and Rat SNCA in Both Primary Hippocampal and Cortical Neurons.** Cortical or hippocampal cultures were prepared from embryonic day 17 (E17) rats. The neurons were treated on DIV 6 with DEX (0, 1, 5 10 uM), (0.1 uM was only assessed in WT cortical neurons). Primary cortical cultures were maintained in FBS-free medium whereas primary hippocampal cultures were maintained in 2.5% FBS enriched medium (see also Table 1, Methods). At 24h post-treatment, cultures were assessed for rat and human SNCA (BAC-rats only) mRNA with Reverse Transcription-PCR. For all experiments *A-F*, results were quantified from RT-PCR and normalized to β-actin. *A-C,* for WT hippocampal rat SNCA mRNA, and BAC hippocampal rat and human SNCA mRNA, two independent experiments were performed in triplicate ($n$=2). DEX treatment did not affect WT hippocampal rat SNCA mRNA levels. 5uM DEX led to a statistically significant increase ($p$=0.0043) of rat SNCA mRNA levels and a non-significant increase in human SNCA mRNA levels in BAC hippocampal neuronal cultures. *D-F,* for WT cortical rat SNCA mRNA, and BAC cortical rat and human SNCA mRNA, four independent experiments were performed in triplicate and two independent experiments were performed in triplicate, respectively ($n$=4, $n$=2, respectively). Results are presented as mean+ S.E.M. *D,* 0.1, 1, 5 and 10 uM DEX led to a statistically significant decrease of rat SNCA mRNA ($p$=0.0039, $p$=0.0286, $p$=0.008, $p$=0.0436, respectively) in WT cortical neuronal cultures. *E, F*. 1uM DEX led to a significant up-regulation ($p$<0.05) of human SNCA mRNA and a non-significant up-regulation of rat SNCA mRNA, in BAC cortical neuronal cultures. (see also

Table 2, summary of results) Statistical analysis was performed via one way ANOVA. *, $p$ <0.05; **, $p$ <0.01; ***, $p$ < 0.0001.

For the aforementioned reasons, a spectrum of DEX doses (0.1, 1, 5 and 10uM) were administered in cultured embryonic rat hippocampal and cortical neurons of WT and hu-AS BAC transgenic rats (BAC), on DIV 6 (early cultures). After 24h of treatment, rat and human SNCA mRNA levels were assessed. At the level of mRNA, we observed increased rat (1.5x, p=0.0043) and a trend for increased hu-SNCA (3x) expression following 5 uM DEX administration in BAC hippocampal neurons as well as increased hu-SNCA expression (2x, p=0.0193) following 1uM DEX with similar trends at higher doses in the BAC cortical neurons, compared to untreated cells (Figure 1B, C, E, F). On the other hand, in WT hippocampal neuronal cultures DEX had no effect on rat SNCA mRNA levels (Figure 1A). In WT cortical neuronal cultures all DEX doses 0.1, 1, 5 and 10uM significantly down-regulated rat SNCA mRNA levels ($p$=0.0039, $p$=0.0286, $p$=0.008, $p$=0.0436, respectively), (Figure 1D). These results show for the first time that DEX administration, particularly the 5 uM dose (BAC hippocampal rat SNCA, p=0.0043), elicits an up-regulation of rat SNCA mRNA (with a similar trend for human) in BAC neurons only, suggesting that stress activation has the potential to exacerbate excess AS burden. Based on the lack of or down-regulation of SNCA expression in WT neuronal cultures, it would appear that stress system activation alone is insufficient to modulate SNCA expression under physiological conditions in the absence of an enhanced alpha-synuclein load. These observations support more broadly the interaction of genetic makeup, i.e. an environment that already hosts the prerequisites to develop this disease, and stress as an environmental factor, may trigger or exacerbate PD pathophysiology in (Gray J.D. et al., 2017).

### Dexamethasone Has Differential effects on AS Protein Levels in Primary Hippocampal Neurons

To further assess the impact of the observed transcriptional up-regulation of SNCA by dexamethasone treatment, we also tested whether DEX induces expression of AS protein levels in our model neuronal culture system by Western Blotting and ELISA. Previous studies using models with the neurotoxin MPTP (Kurkowska-Jastrzebska et al., 2004), WT neuronal cultures or cell lines (Park J.M et al, 2013) have identified either DEX-driven neuroprotection or increased AS expression; these studies have stressed the importance of using the appropriate DEX dosage, as well as highlighted the limitation of their animal models or cell lines to recapitulate the gene-environment interaction in PD pathogenesis.

The hu-AS BAC-transgenic rat model (BAC rat model) of PD overexpresses full-length human AS, which occurs in familial PD. In addition, the transgenic construct contains all the necessary regulatory elements surrounding the SNCA gene, and thus, stimuli that normally affect human AS expression, are expected to

do so in this model. To this end, cultured embryonic rat hippocampal neurons were treated on DIV 6 with DEX (0, 0.1, 1, 5, 10 uM) and total AS protein levels as well as levels of serine 129 phosphorylated AS (p129 AS) were measured with Western Blotting and ELISA 48h after DEX treatment.

From the Western immunoblot analysis we found that 1uM DEX increases endogenous rat AS expression in the Triton-X-soluble (2x, p=0.0386) and the SDS-soluble fraction (3.5x, p=0.096), while higher doses did not affect AS levels in WT cultured hippocampal neurons (Figure 2A, C). pAS levels did not change (Figure 2B). Contradictory results were obtained when the Triton-X-soluble proteins were analyzed with ELISA for total AS expression levels. In this case, it seems there is a slight, but not significant, increase in AS levels at 1 uM DEX (Figure 2F). Overall, the results are not entirely consistent using the two different methods and thus requires more replicates and further investigation. When the SDS soluble fraction of WT hippocampal proteins was analyzed with ELISA, there was a significant increase (3.5x, p=0.096) in a-synuclein protein levels following 1 uM DEX treatment (Figure 2H). The same trend was identified by Western Immunoblotting but more replicates need to be done to corroborate the results with this method (Figure 2D). With respect to BAC hippocampal neurons, no differences in either total AS or p129-AS in the Triton-X-soluble cytosolic fraction were observed following DEX treatment (Figure 3A-D). With respect to the SDS-soluble fraction, total AS protein levels remained unchanged or decreased with 10 uM (0.5x, p=0.0416) DEX in BAC hippocampal neurons; however, there was a significant increase (1.25x, p=0.0042) of p129 AS following 5uM DEX treatment- the same dose that upregulated AS gene transcription (Figure 3E-G).

Together, these results suggest that in WT hippocampal cultures specific DEX doses may increase AS levels, but the data are not entirely consistent and more experiments need to be done to corroborate this. Concerning the BAC hippocampal cultures; overall it seems there is no effect on AS levels upon DEX treatment, except a mild effect only with one DEX dose (5uM) on pAS levels in the SDS-soluble material that needs further investigation.

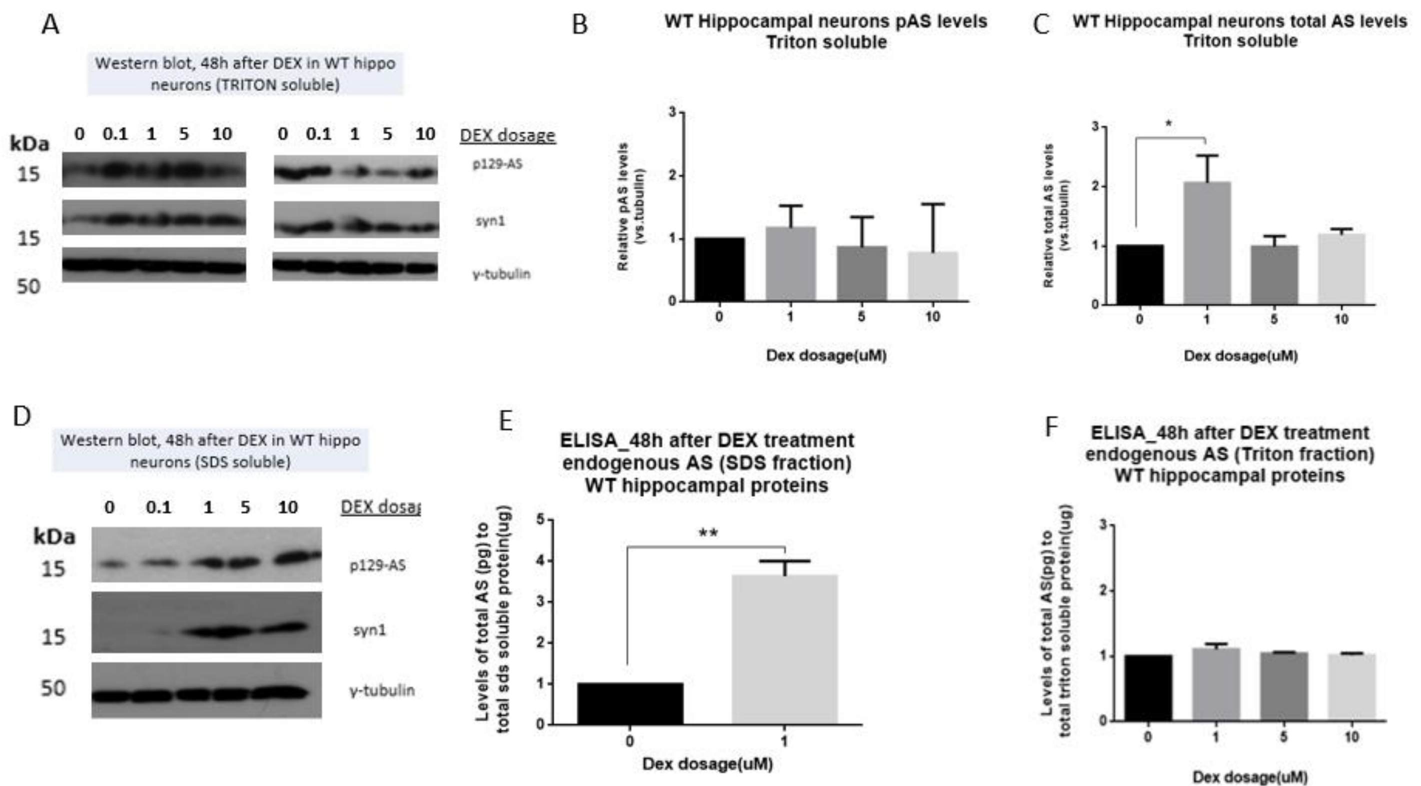


**Figure 2. Dexamethasone has differential effects on AS protein levels in WT primary hippocampal neurons.** WT hippocampal cultures were prepared from embryonic day 17 (E17) rats. The neurons were treated on DIV 6 with DEX (0, 1, 5 10uM), (0.1uM DEX was only assessed in one experiment and it is not included in quantification of results). The primary neuronal cultures were maintained in 2.5% FBS enriched medium (see also Table 1, Methods). *A, D,* At 48h post-treatment, total a-synuclein (AS) and pSER129-a-synuclein (pAS) protein levels (both Triton-X and SDS soluble) were assessed with Western Blotting. γ-tubulin was used as a loading control. *B, C,* Quantification of Western blots using ImageJ and statistical analysis using GraphPad Prism 6. *E, F,* At 48h post-treatment, AS protein levels (Triton-X soluble) or (SDS soluble) were assessed with ultra-sensitive ELISA for AS. The AS protein levels expressed in pictograms (pg) were normalized with total protein level (Triton-X soluble or SDS soluble, respectively) of the cell lysates per condition expressed in micrograms (ug). *A-C*, F, for WT hippocampal total AS and pSER129-a-synuclein (pAS) protein levels (Triton-X soluble) two independent experiments were performed in triplicate for Western immunoblotting and the same samples were used for ELISA (*n*=6). *D,* for WT hippocampal total AS and pSER(129)-a-synuclein (pAS) protein levels (SDS soluble) one experiment was performed in triplicate for Western immunoblotting. Results are presented as means S.E. (*n*=3). *E,* for WT hippocampal AS protein levels (SDS soluble) two independent experiments were performed in triplicate and duplicate, respectively, for ELISA. Results are presented as means S.E. (*n*=5). *A, C,* 1uM DEX led to a statistically significant increase (p=0386) of total Triton-soluble AS in WT hippocampal neurons but ELISA, *D*, did not provide the same robust effect of DEX. *B,* DEX did not affect pAS protein levels (Triton-X soluble) *E*, 1uM DEX led to a statistical significant increase (p=0.0096) of SDS-soluble AS in WT hippocampal neurons. (see also Table 2, summary of results). Results are presented as mean ± S.E.M. Statistical analysis was performed via one-way ANOVA test with Bonferroni's correction. *, p< 0.05; **, p< 0.01; ***, p< 0.0001.

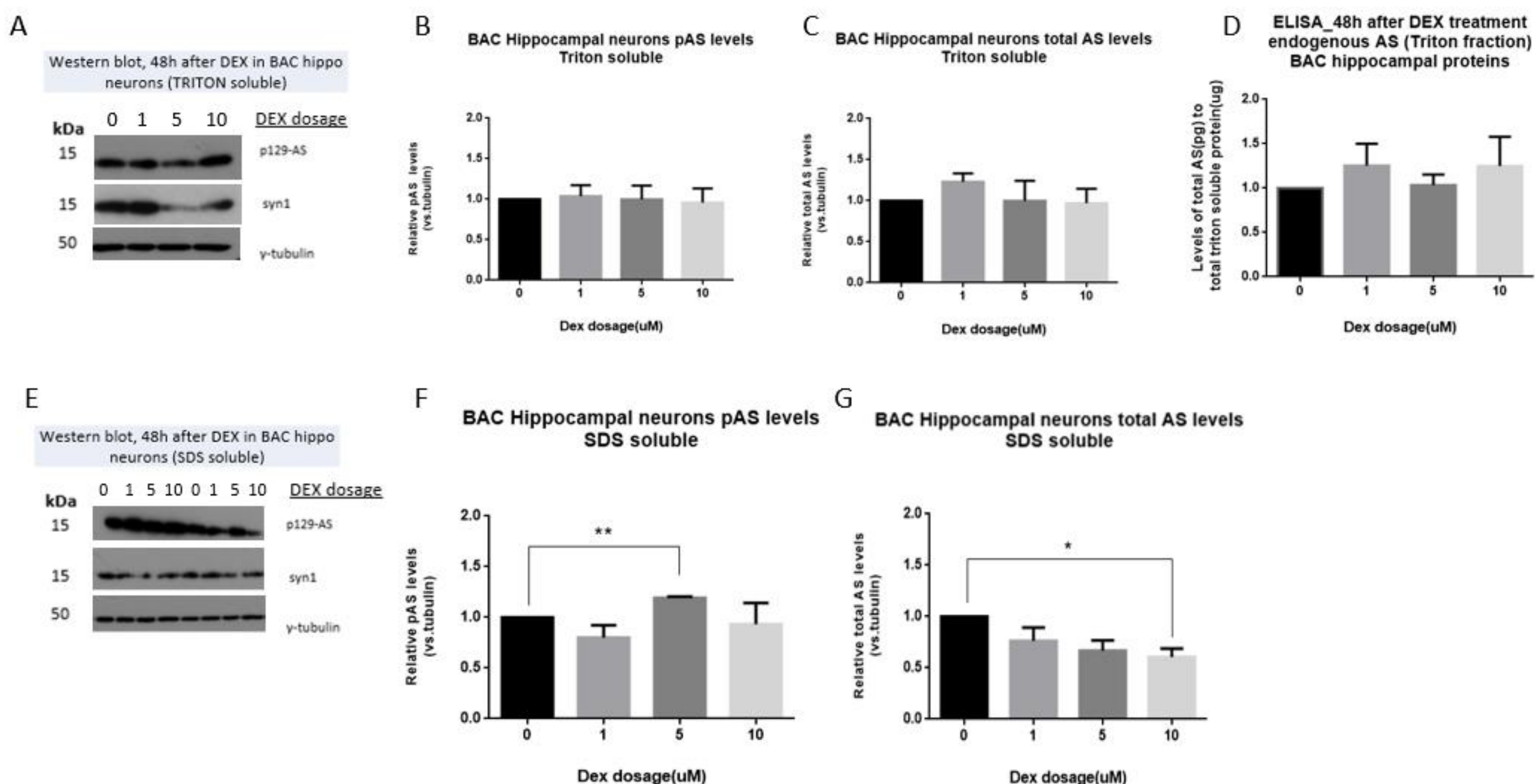


**Figure 3. Dexamethasone has differential effects on AS protein levels in BAC-transgenic primary hippocampal neurons.** BAC-transgenic hippocampal cultures were prepared from embryonic day 17 (E17) rats. The neurons were treated on DIV 6 with DEX (0, 1, 5 10uM). The primary neuronal cultures were maintained in 2.5% FBS enriched medium (see also Table 1, Methods). *A, E,* At 48h post-treatment total a-synuclein (AS) and pSER(129)-a-synuclein (pAS) protein levels (both Triton-X and SDS soluble) were assessed with Western Blotting. γ-tubulin, loading control. *B, C, F, G,* Quantification of Western blots using ImageJ and statistical analysis using GraphPad Prism 6. *D,* At 48h post-treatment AS protein levels (Triton-X soluble) were assessed with ultra-sensitive ELISA for AS. The AS protein levels expressed in picograms(pg) were normalized with total protein level (Triton-X soluble) of the cell lysates per condition expressed in micrograms (ug). *A-G,* for BAC hippocampal total a-synuclein (AS) and pSER(129)-a-synuclein (pAS) protein levels (Triton-X soluble and SDS soluble) two independent experiments were performed in triplicate for Western blotting and ELISA. ($n$=2 and $n$=2, respectively). *F,* 5uM DEX led to a statistical significant increase of SDS-soluble pAS (p=0.0042) in BAC hippocampal neurons. *G*, 10uM DEX led to a statistically significant decrease (p=0.0416) of total AS in BAC hippocampal neurons. Also, DEX doses 1 and 5uM led to decreased levels of total AS protein levels but this was not statistically significant. B, DEX doses had no effect in pAS protein levels (Triton-X soluble) in BAC hippocampal neurons. C, D, 1uM DEX led to a slight increase in total AS protein levels (Triton-X soluble) but this was not statistically significant (same trend but not statistically significant, for AS ELISA). (see also Table 2, summary of results). Results are presented as mean± S.E.M. Statistical analysis was performed via an unpaired $t$ test. *, $p$ <0.05; **, $p$ <0.01; ***, $p$ <0.0001.

## Dexamethasone Has Differential effects on AS Protein Levels in Primary Cortical Neurons

In addition to hippocampal neurons, we also assessed the modulation of AS protein levels in cultured primary cortical neurons 48h and 72h after DEX treatment (1, 5, 10uM) to track the possible consequences of the up-regulation of AS transcription under these conditions but also evaluate the possibility of direct effects of DEX on AS protein expression and accumulation.

In WT cortical neurons, we did not detect any change increase in total or p129 AS protein levels following DEX treatment in the Triton-X-soluble material, but rather a decrease in the 10uM (0.5x, p=0.0119) and 5uM (0.5x, p=0.0113) doses, respectively (Figure 4A-C ). On the other hand, in the SDS-soluble fraction, there was a statistically significant increase (1.75x, p=0.0274) of total AS protein levels with 1uM DEX treatment whereas DEX had no statistically significant effect in p129 AS protein levels (Figure 4D-F). Conversely, in the BAC model, neither the Triton-X-soluble fraction nor the SDS-soluble fraction revealed any statistically significant differences in total or p129 AS protein levels following all doses of DEX treatment tested (Figure 5A-F).

Due to the fact that protein levels of a-synuclein were analyzed at 48h after DEX treatment and no statistical significance arose in AS expression compared to the significant up-regulation of AS mRNA that we identified at 24h after DEX treatment, specifically at 5uM DEX of human AS mRNA of BAC cortical neurons (Figure 1F), we proceeded with the investigation of a potential time-dependent effect of AS protein level modulation with DEX in cultured cortical neurons 72h after DEX treatment (Supplementary Figure 1). At the level of mRNA, in WT cortical neurons, there is a trend of increase in rat SNCA mRNA at 0.1 and 5uM DEX dose compared to untreated cells whereas, in BAC cortical neurons, there is an increase in human SNCA mRNA at 1uM DEX dose and a down regulation of rat SNCA mRNA compared to untreated cells . These results are preliminary but showed the same trend in human SNCA mRNA as the BAC cortical neurons showed 48h after DEX treatment. However, in WT cortical neurons we observed for the first time an increase of rat SNCA mRNA level at 72h after DEX treatment (preliminary results) (Supplementary Figure 1A) compared to the absence of up regulation at 24h after treatment with the same DEX doses (figure 1).

At the protein level, our preliminary results show that WT cortical neurons exhibited an increase in total AS protein levels from 1uM to 10uM but this was not statistically significant, and DEX had no effect in pAS levels in Triton and SDS soluble fraction (Supplementary Figure 1B, C). In BAC cortical cultures, DEX did not affect the levels of pAS and total AS in the Triton soluble fraction. On the other hand a trend of

increased pAS and total AS levels (SDS soluble) was observed at 1 and 5uM DEX, respectively, but not statistically significant (Supplementary Figure 1D, E).

Together these results suggest that in WT cortical neurons there may be an increase in AS levels in the SDS-soluble material for a specific DEX dose (1uM). On the other hand, DEX seems to have no effect in BAC cortical cultures. As it concerns the 72h time point, it may be a delayed effect on transcription of SNCA in WT cultures; however, a complete time course with more experiments is needed to address this point.

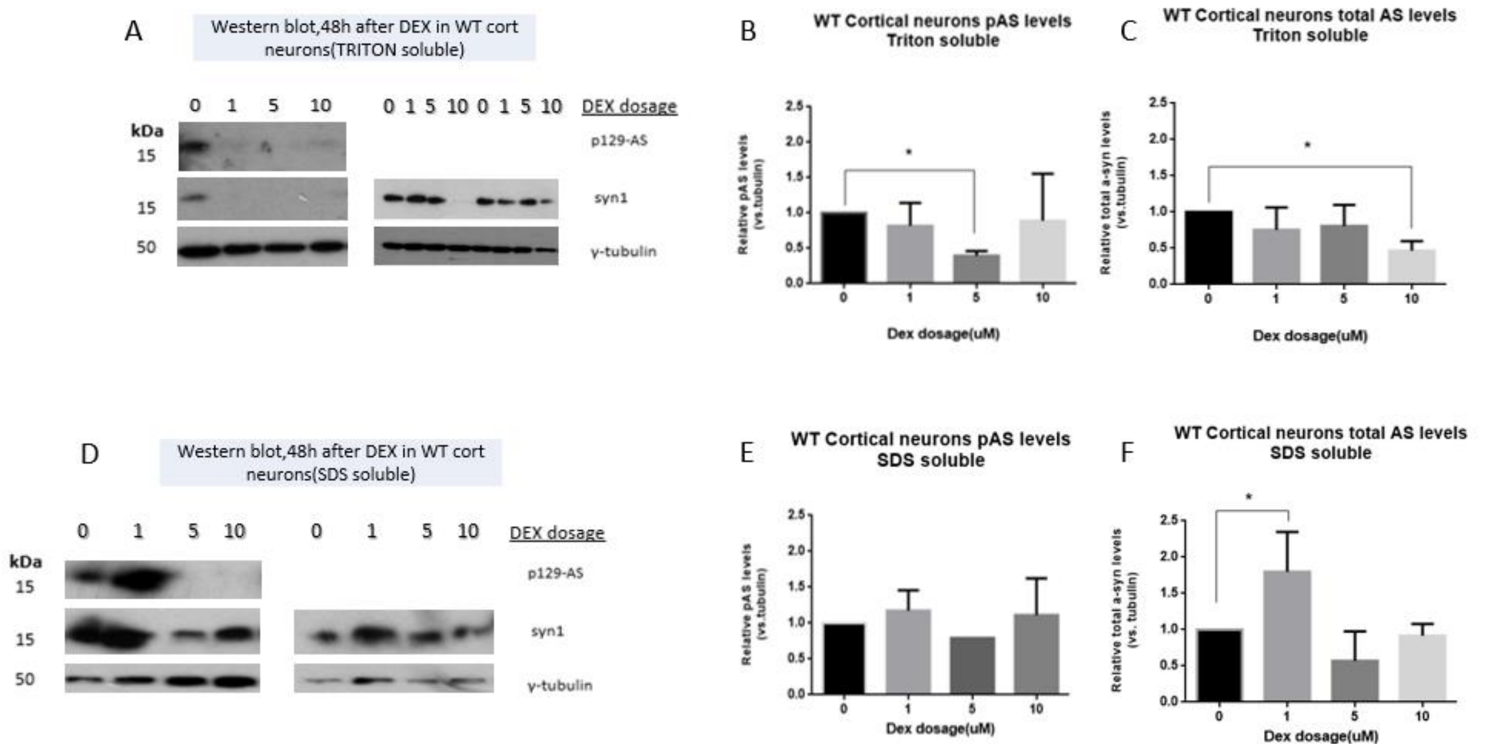


**Figure 4. Dexamethasone has differential effects on AS protein levels in WT primary cortical neurons.** WT cortical cultures were prepared from embryonic day 17 (E17) rats. The neurons were treated on DIV 6 with DEX (0, 1, 5 10uM). The primary neuronal cultures were maintained in 0 or 2.5% FBS enriched medium, data showed same trend and pooled (see also Table 1, Methods). *A, D,* At 48h post-treatment total a-synuclein (AS) and pSER(129)-a-synuclein (pAS) protein levels (both Triton-X and SDS soluble) were assessed with Western Blotting. γ-tubulin, loading control. *B, C, E, F,* Quantification of Western blots using ImageJ and statistical analysis using GraphPad Prism 6. *A-F,* for WT cortical total a-synuclein (AS) and pSER(129)-a-synuclein (pAS) protein levels (Triton-X soluble and SDS soluble, respectively) three independent experiments were performed in triplicate and two independent experiments were performed in triplicate for Western blotting, respectively. ($n$=3 and $n$=62, respectively). A-C, Efficient decrease of pAS and total AS protein levels (Triton-X soluble) at 5 and 10uM DEX, respectively, led to a statistical significant decrease (p=0.0113, p=0.0119, respectively) in WT cortical neurons. *F,* Efficient overexpression of total AS protein levels (SDS soluble) at 1uM DEX led to a statistical significant increase

(p=0.0274) in WT cortical neurons. *E,* no effect of DEX at pAS protein levels (SDS soluble) in WT cortical neurons. (see also Table 2, summary of results) Results are presented as mean + S.E.M. Statistical analysis was performed via an unpaired *t* test and multiple comparisons with one-way ANOVA test with Bonferroni's correction. *, *p*<0.05; **, *p*<0.01; ***, *p*<0.0001.

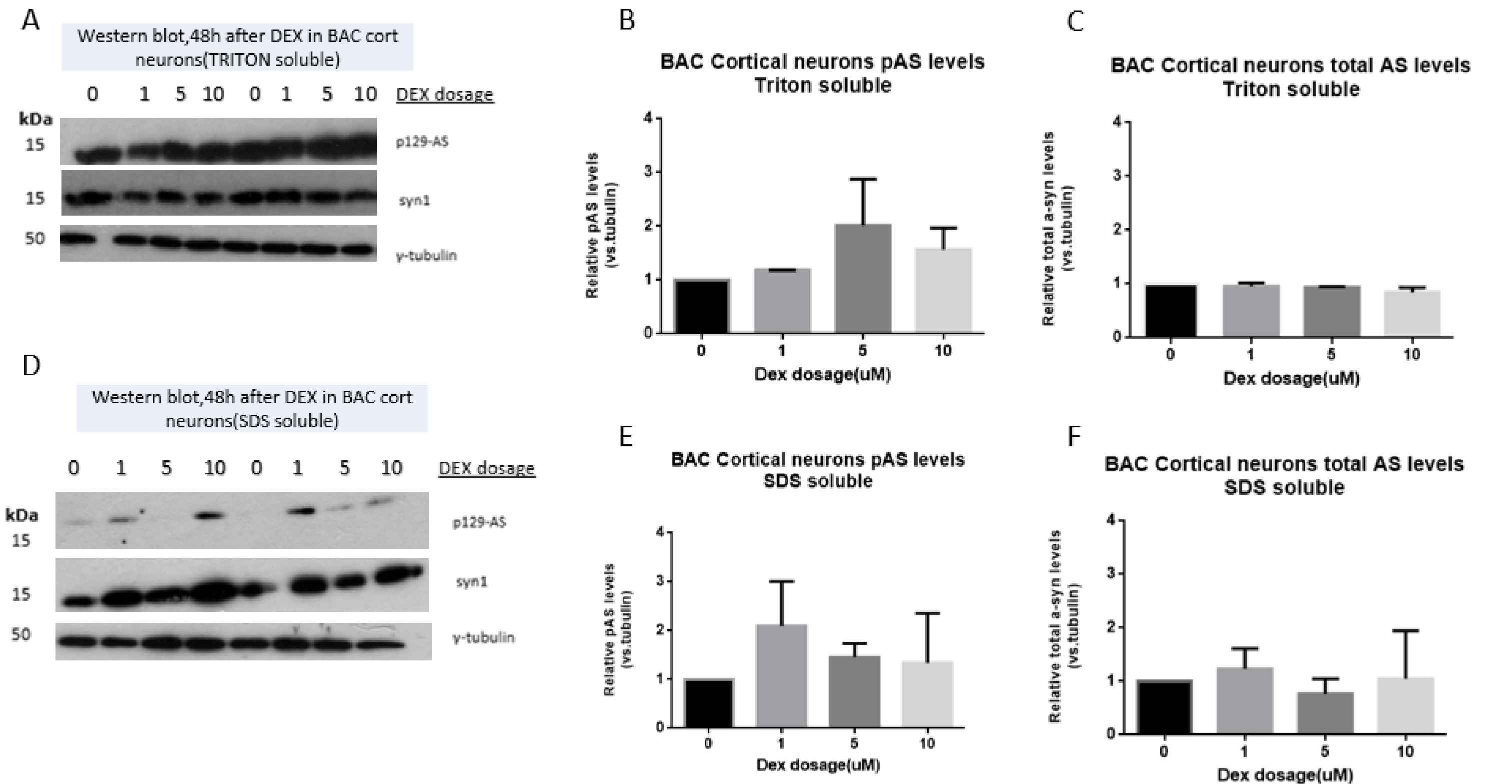


**Figure 5. Dexamethasone has no differential effects on AS protein levels in BAC primary cortical neurons.** BAC-transgenic cortical cultures were prepared from embryonic day 17 (E17) rats. The neurons were treated on DIV 6 with DEX doses (0, 1, 5 10uM). The primary neuronal cultures were maintained in 0 or 2.5% FBS enriched medium, data showed same trend and pooled (see also Table 1, Methods). *A, D,* At 48h post-treatment total a-synuclein (AS) and pSER(129)-a-synuclein (pAS) protein levels (both Triton-X and SDS soluble) were assessed with Western Blotting. γ-tubulin, loading control. *B, C, E, F,* Quantification of Western blots using ImageJ and statistical analysis using GraphPad Prism 6. *A-F*, for BAC cortical total a-synuclein (AS) and pSER(129)-a-synuclein (pAS) protein levels (Triton-X soluble and SDS soluble, respectively) two independent experiments were performed in triplicate for Western blotting, respectively. (*n*=2 and *n*=2, respectively). *A-C*, 1uM DEX led to a slight increase in pAS protein levels (Triton-X soluble) but not statistical significant, but DEX doses had no effect in total AS protein levels (Triton-X soluble) in BAC cortical neurons. *D-F,* 1 and 5uM DEX led to a slight increase in pAS protein levels (SDS soluble) but not statistical significant, but DEX doses had no effect in total AS protein levels (SDS soluble) in BAC cortical neurons. (see also Table 2, summary of results) Results are presented as mean + S.E.M. Statistical analysis was performed via an unpaired *t* test and multiple comparisons with one-way ANOVA test with Bonferroni's correction *, *p*<0.05; **, *p* <0.01; ***, *p* <0.0001.

## Absence of Dexamethasone effect on AS Secretion in Primary Hippocampal and Cortical Neurons

AS is mainly expressed by neuronal cells and is generally considered to exist as a cytoplasmic protein. Several studies have been reported the unexpected presence of AS in conditioned culture media from non-transfected and AS-transfected human neuroblastoma cells, as well as in human cerebrospinal fluid and blood plasma (El-Agnaf et al., 2003, Emmanouilidou et al., 2011). These data suggest that the protein may be released from cells via a non-classical secretory pathway and may therefore, exert paracrine effects in the extracellular environment. These findings elicited our motivation to further track the consequences of DEX treatment on the potential secretion of AS into the cultured cells' conditioned media.

For this reason, we utilized an ultra-sensitive ELISA for AS generated in the lab (Emmanouilidou et al., 2011) to assess the levels of secreted total AS (human and rodent) levels in conditioned culture media from WT and BAC cortical and hippocampal neuronal cultures. For this purpose, we collected the conditioned media from all time points (24, 48 and 72h) of experiments described above. This way we were able to follow the pattern of AS secretion into the extracellular space by DEX treatment compared with mRNA and intracellular protein levels in order to reach a conclusion about the DEX-dependent regulation of AS in our model neuronal culture system.

A previous study by Reyers et al., 2005, showed for the first time dopaminergic neurons derived from induced pluripotent stem cells (iPSCs) acquired from biopsies isolated from genetically healthy controls or from biopsies carrying a triplication of the AS gene that leads to familial PD (Singleton et al.,2003) secrete AS that can be taken up by neighboring neurons. Both types of neurons secreted AS but as expected, familial PD neurons expressed higher AS levels compared to normal AS-expressing neurons. Thus, we sought to identify potential differences in AS secretion in our BAC primary neuronal culture model following DEX treatment.

In the conditioned media of WT hippocampal and cortical neurons, at 24h after DEX treatment there were no observed differences in the amount of secreted AS, except a slight increase in 1um DEX but not statistically significant (Figure 6A,C). On the other hand, in the conditioned media of BAC hippocampal neurons, DEX treatment showed no significant change in the levels of secreted AS compared to untreated cells (Figure 6B, D). There was an observed decrease (0.5x, p=0.0174) in AS secretion following 10 uM DEX in the cortical neuron conditioned media of BAC neurons, which requires further investigation (Figure 6D).

It is likely that 24h after treatment may be too early for the cells to react to transcriptional AS regulation and express as well as secrete detectable changes of AS.

In addition, we performed ELISA for AS of conditioned media collected at 48h after DEX treatment from cultured WT and BAC hippocampal and cortical neurons (Figure 7, 8). In this case we expressed the results total pg of secreted AS as arbitrary units (AU) as above, as well as total pg of secreted AS normalized to total ug of endogenous protein amount (obtained from protein measurements of Triton-X-soluble fraction) per condition, and as the ratio of total pg of secreted AS to total pg levels of endogenous AS (obtained from ELISA measurements of of total AS of Triton–X-soluble proteins) per condition. With this approach, we were able to assess primarily the absolute values of secreted AS in terms of the same amount of cells per condition of DEX treatment and also, to evaluate the difference between the protein level of endogenous AS and secreted AS per condition.

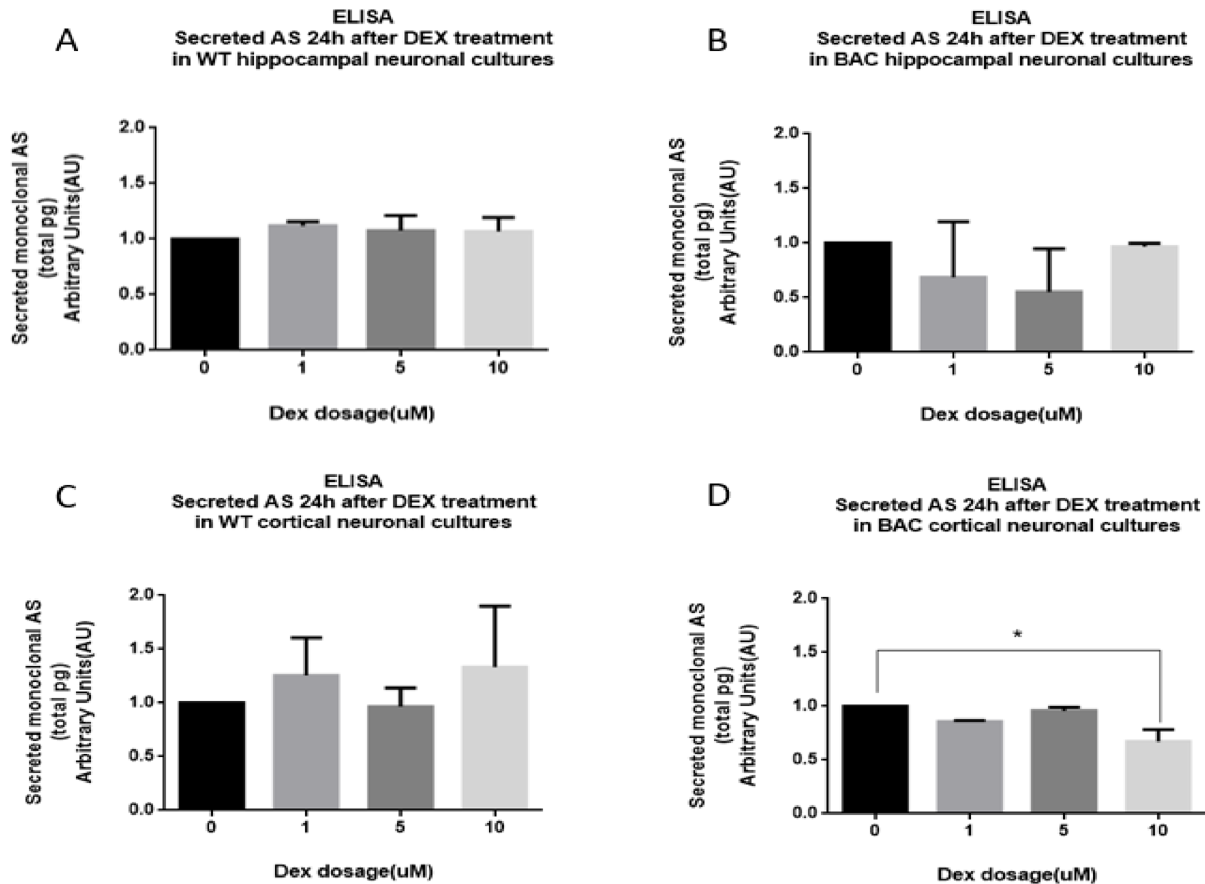


**Figure 6. Dexamethasone has moderate effects on secreted AS protein levels in WT and BAC cortical and hippocampal cultured condition media.** WT and BAC-transgenic hippocampal and cortical neuronal cultures were prepared from embryonic day 17 (E17) rats. The neurons were treated on DIV 6 with DEX doses (0, 1, 5 10uM). The primary neuronal cultures were maintained in 2.5% for hippocampal and 0 or

2.5% FBS enriched medium for cortical cultures (data showed same trend and pooled), (see also Table 1, Methods). At 24h post-treatment cultures were assessed for SNCA mRNA levels (see also figure.1), and the condition media of the cultures were collected at that time-point. The cultured condition media were assessed for secreted a-synuclein (AS) protein levels utilizing ultra-sensitive ELISA for monoclonal AS. Secreted AS protein levels are expressed as pictograms (pg) but were not normalized (arbitrary units). *A-D,* for WT and BAC hippocampal and cortical cultured condition media secreted AS protein levels two independent experiments were performed in triplicate and two independent experiments were performed in triplicate, respectively. (n=2, n=2, n=2, n=2 respectively). *A*, 1uM DEX led to a slight increase in secreted AS protein levels, but not statistical significant, in WT cultured hippocampal condition media. *B*, DEX doses had no effect in secreted AS protein levels in BAC cultured hippocampal condition media. C, DEX doses had no effect in secreted AS protein levels in WT cultured cortical condition media. D, 1uM DEX led to a slight decrease in secreted AS protein levels, but not statistical significant, and 10uM DEX led to a statistical significant decrease (p=0.0174) in secreted AS protein level in BAC cultured cortical condition media. (see also Table 2, summary of results) Results are presented as mean $\pm$ S.E.M. Statistical analysis was performed via one-way ANOVA test with Bonferroni's correction. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.0001$.

In WT hippocampal conditioned media, at 48h after DEX treatment there is a slight increase of the amount of secreted AS at 10uM DEX, but this is not significant (Figure 7A, B). In BAC hippocampal conditioned media of the 48h time point, the same trend is observed, at 1uM there is an increase in secreted AS, but not statistically significant, compared to base line conditions (Figure 7D,E). The same pattern was identified for conditioned media collected from WT and BAC cortical neurons, but again differences were not significant (Figure 8). Interestingly, the ratio of secreted AS to endogenous AS , both in WT and BAC cultured hippocampal neurons, was not affected by the DEX doses but showed the same trend of secreted levels of AS at 48h (figure 7C, F).

When we compared WT versus BAC hippocampal neurons' condition media, we identified that in untreated cells the level of secreted AS is significantly higher (2x, p<0.05) in BAC neurons than in WT, as we speculated (Figure 9). In addition, in all DEX doses there is a statistically significant increase (2.5x, p<0.01) of secreted AS in BAC compared to WT neuronal conditioned media. The same results aroused from cultured cortical condition media (Figure 9).

Together these results suggest that BAC neuronal cultures secrete more AS than WT neuronal cultures independent of DEX treatment (Figure 7-9). The above findings indicate that there is lack of a DEX-dependent effect on AS secretion in both WT and BAC neuronal cultures.

However, we need to underline that our ultra-sensitive ELISA method is restricted to the detection of total AS, giving us no information about oligomeric or fibrilar levels of AS (if they have been formed) neither intracellularly nor extracellularly. It is significant to add that at the cytosolic protein level, we detect statistically significant increases of total AS and p129 AS in the SDS-soluble fraction of proteins (Figure 3, 4). This fraction represents the less soluble forms of AS, in our case representing membrane-associated AS, which may, if in some way, be secreted from the cell either in exosome-like vesicles or as bare oligomers (El-Agnaf et al, 2003). If this is the case, such forms cannot be detected with our specific method of ELISA.

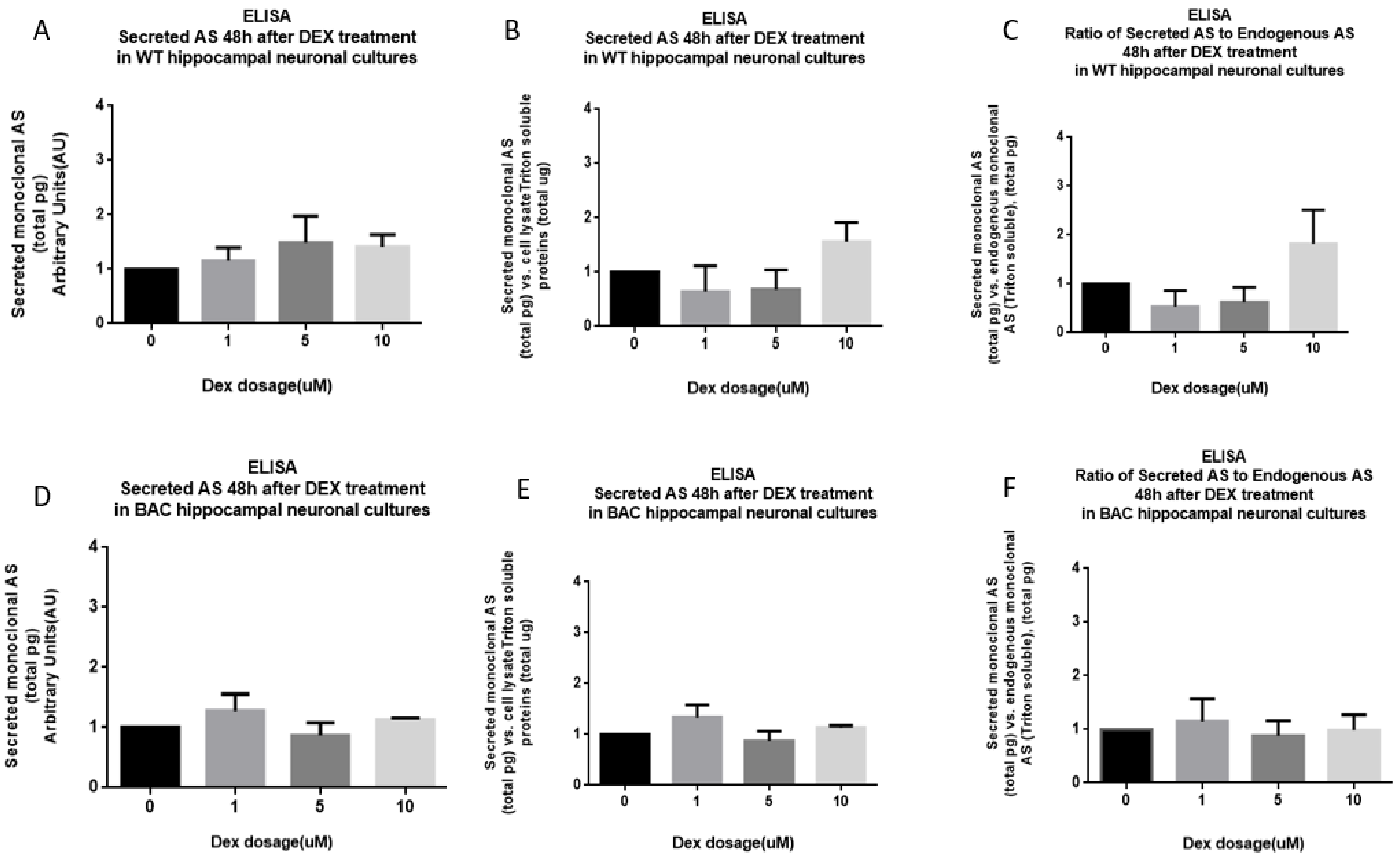


**Figure 7. Dexamethasone has moderate effects on AS secretion in WT and BAC-transgenic hippocampal neuronal cultures.** WT and BAC-transgenic hippocampal neuronal cultures were prepared from embryonic day 17 (E17) rats. The neurons were treated on DIV 6 with DEX doses (0, 1, 5 10uM). The primary neuronal cultures were maintained in 2.5% FBS enriched medium (see also Table 1, Methods). At 48h post-treatment, cultures were assessed for endogenous AS protein levels (see also figure.1, 2), and the condition media of the cultures were collected at that time-point. The cultured condition media were assessed for secreted a-synuclein (AS) protein levels utilizing ultra-sensitive ELISA for monoclonal AS. *A,D,* Secreted AS protein levels are expressed as pictograms(pg) but were not normalized (arbitrary units). *B,E,* Secreted AS protein levels are expressed as pictograms(pg) and normalized with total protein levels of cell

lysates (Triton-X soluble) expressed in micrograms(ug) per condition. *C,E,* monoclonal AS protein levels are expressed as the ratio of secreted AS (pg) to endogenous AS (pg) which levels are also obtained from ELISA for the Triton-X soluble protein fraction (see also figure 2, 3). *A-F*, for WT and BAC hippocampal cultured condition media secreted AS protein levels, two independent experiments were performed in triplicate and two independent experiments were performed in triplicate, respectively. (n=2, n=2, respectively). *B*, not a dex-dependent effect was observed in secreted levels of AS *C*, 1 and 5uM DEX led to a decrease of secreted AS protein levels compared to endogenous AS protein levels, respectively, but not statistical significant, in WT hippocampal neuronal cultures. *E*, no Dex-dependent effect in secreted AS except a trend of increase in 1uM DEX but not statistically significant *F*, not a Dex-dependent effect observed in the ratio of secreted to endogenous AS (see also Table 2, summary of results) Results are presented as mean $\pm$ S.E.M. Statistical analysis was performed via an unpaired *t* test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.0001$.

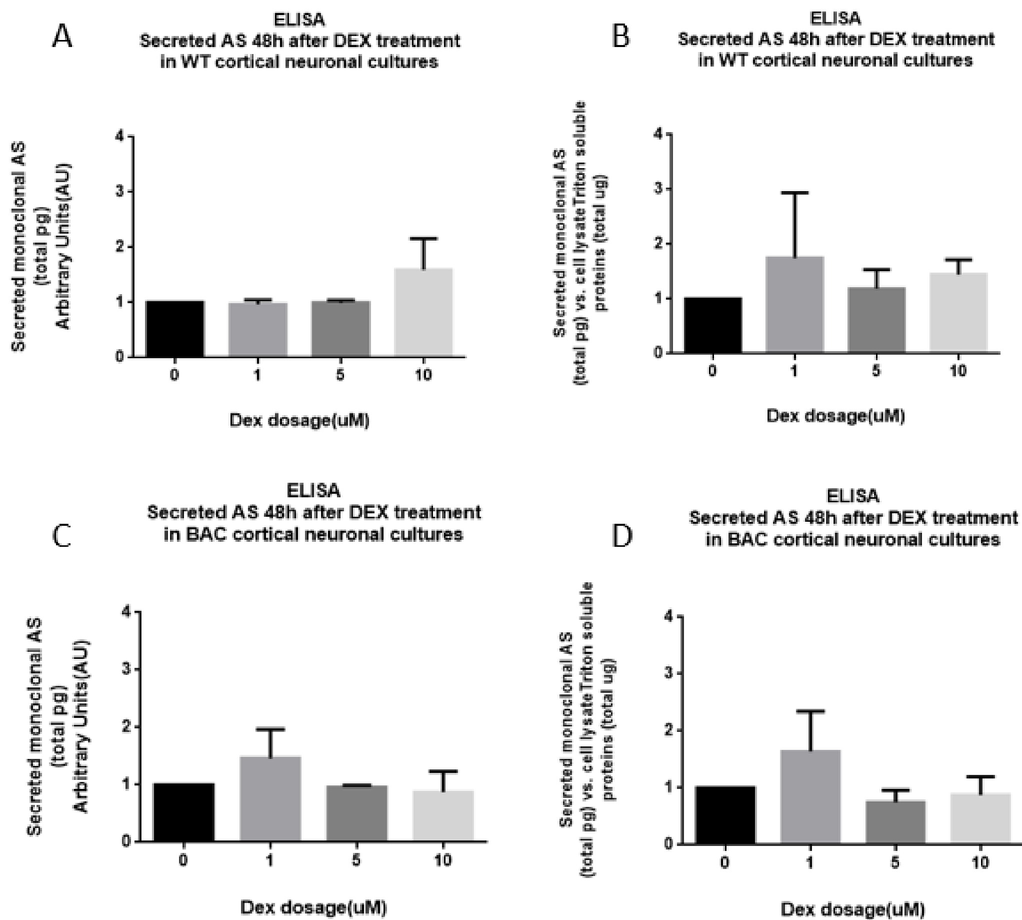


**Figure 8. Dexamethasone does not affect AS secretion in WT and BAC-transgenic cortical neuronal cultures.**

WT and BAC-transgenic cortical neuronal cultures were prepared from embryonic day 17 (E17) rats. The neurons were treated on DIV 6 with DEX doses (0, 1, 5 10uM). The primary neuronal cultures were maintained in 2.5% FBS enriched medium (see also Table 1, Methods). At 48h post-treatment, cultures were assessed for total AS protein levels with Western blotting (see also figure.4, 5), and the condition

media of the cultures were collected at that time-point. The cultured condition media were assessed for secreted a-synuclein (AS) protein levels utilizing ultra-sensitive ELISA for monoclonal AS. *A,C,* Secreted AS protein levels are expressed as pictograms (pg) but were not normalized (arbitrary units). *B, D,* Secreted AS protein levels are expressed as pictograms (pg) and normalized with total protein levels of cell lysates (Triton-X soluble) expressed in micrograms (ug) per condition. *A-D,* for WT and BAC cultured cortical condition media secreted AS protein levels, two independent experiments were performed in triplicate and two independent experiments were performed in triplicate, respectively. (*n=2, n=2*, respectively). *A,B,* DEX had no effect in secreted AS protein levels in WT cortical neuronal cultures. *C,D,* DEX had no effect in secreted AS protein levels in BAC cortical neuronal cultures. (see also Table 2, summary of results) Results are presented as mean + S.E.M. Statistical analysis was performed via an unpaired *t* test. *, *p* <0.05; **, *p* <0.01; ***, *p* <0.0001.

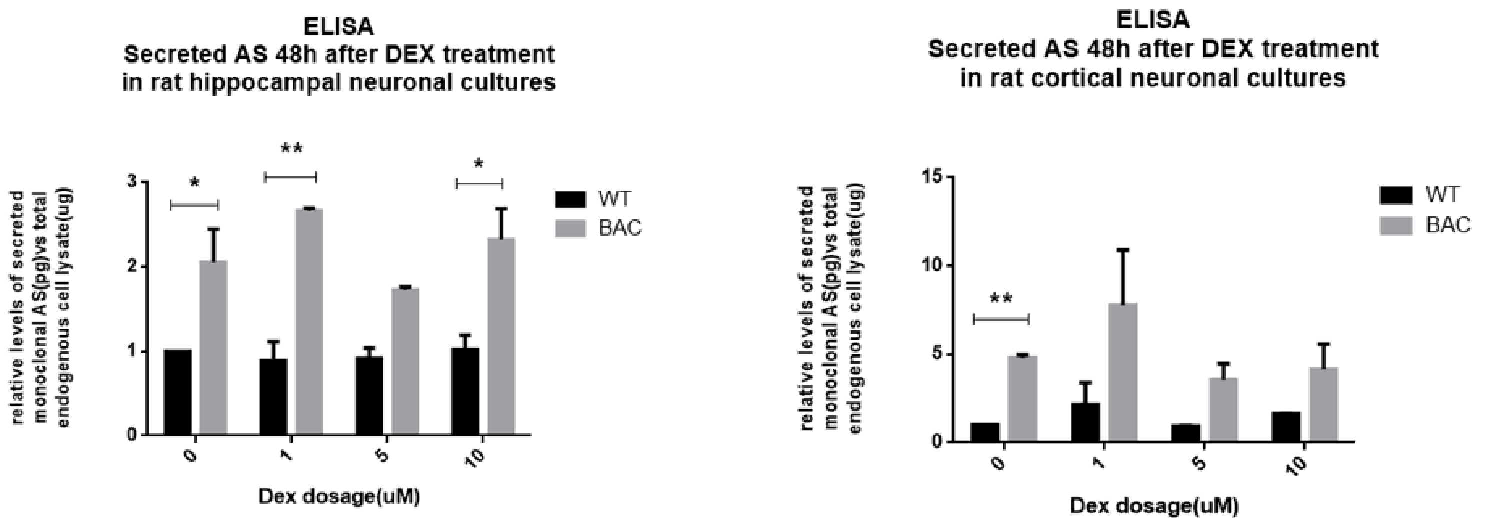


**Figure 9. Dexamethasone has differential effects in secreted AS between WT and BAC-transgenic primary neuronal cultures.**

WT and BAC-transgenic hippocampal and cortical neuronal cultures were prepared from embryonic day 17 (E17) rats. The neurons were treated on DIV 6 with DEX doses (0, 1, 5 10uM). The primary neuronal cultures were maintained in 2.5% FBS for hippocampal and 2.5% FBS enriched medium (see also Table 1, Methods). At 48h post-treatment, cultures were assessed for total AS protein levels with Western blotting and ELISA (see also figure 2, 3, 4, 5), and the condition media of the cultures were collected at that time-point. The cultured condition media were assessed for secreted a-synuclein (AS) protein levels utilizing ultra-sensitive ELISA for monoclonal AS. Secreted AS protein levels are expressed as pictograms (pg) and normalized with total protein levels of cell lysates (Triton-X soluble) expressed in micrograms (ug) per condition. A, B, for WT and BAC cultured hippocampal and cortical condition media secreted AS protein levels, two independent experiments were performed in triplicate and two independent experiments were performed in triplicate, respectively. (*n=2, n=2, n=2, n=2*, respectively). (see also figure 7, 8) . Utilizing two-way ANOVA for multiple comparisons, we compared WT and BAC cultured conditioned media secreted AS protein levels in hippocampal and cortical neuronal cultures, respectively. *A,* At control conditions (0uM DEX), secreted AS protein levels are higher (p<0.05) in BAC compared to WT hippocampal condition media. 1 and 10uM DEX led to statistical significant increase (*p*<0.05, *p*<0.01, respectively) and 5uM led to an increase but not statistical significant, in secreted AS protein levels in BAC compared to WT hippocampal condition media, respectively. *B*, At control conditions (0uM DEX), secreted AS protein levels are higher (*p*=0.0047) in BAC compared to WT cortical condition media. 1, 5 and 10uM DEX led to increase

in secreted AS protein levels, but not statistical significant, in BAC cortical condition media compared to WT cortical condition media, respectively. Results are presented as mean ± S.E.M. Statistical analysis was performed via two-way ANOVA test for multiple comparisons with Bonferroni's correction *, $p<0.05$; **, $p<0.01$; ***, $p<0.0001$.

| *SNCA* mRNA levels | | | | |
|---|---|---|---|---|
| Region | Hippocampus | | Cortex | |
| mRNA | Human | Rat | Human | Rat |
| WT | N/A | - | N/A | ↓[0.1,5]uM**,[1,10]uM* |
| BAC | - | ↑5uM** | ↑1uM* | - |

| Cytosolic Protein Level | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Region | Hippocampus | | | | Cortex | | | |
| Protein | pAS | | Total AS | | pAS | | Total AS | |
| fraction | Triton-X | SDS | Triton-X | SDS | Triton-X | SDS | Triton-X | SDS |
| WT | - | - | ↑1uM* | ↑1uM**(1) | ↓5uM* | - | ↓10uM* | ↑1uM* |
| BAC | - | ↑5uM** | - | ↓[(all/n.s),10*] | - | - | - | - |

| Secreted Monoclonal AS | | | | |
|---|---|---|---|---|
| Region | Hippocampus | | Cortex | |
| Hours after DEX | 24 | 48 | 24 | 48 |
| WT | - | - | - | - |
| BAC | - | - | ↓[1(n.s),10uM*] | - |

| Secreted AS / Endogenous AS | |
|---|---|
| Region | Hippocampus |
| Hours after DEX | 48 |
| WT | ↓[1,5uM](n.s) |
| BAC | ↓[1*,5(n.s)uM] |

**Table 2. Summary of dexamethasone effects on transcription, expression and secretion of AS in primary neuronal cultures.** This table summarizes the effects of Dexamethasone treatment in WT and BAC-transgenic hippocampal and cortical primary neuronal cultures (figures 1-8). SNCA mRNA levels were assessed at 24h post-treatment with Reverse Transcription-PCR. Cytosolic total AS and pSER(129)-AS protein levels were assessed at 48h post-treatment with Western Immunoblotting. (1) Total endogenous monoclonal AS protein levels (SDS soluble) measured with ELISA. Secreted monoclonal AS was measured with ELISA for 24h and 48h post-treatment cultured condition media. Secreted to Endogenous monoclonal AS protein levels were measured with ELISA, for 48h post-treatment cultured condition media. Abbreviations: N/A (not applicable), n.s. (not statistical significant), all (includes all dex doses). Statistical

analysis was performed via an unpaired *t* test and multiple comparisons with one-way ANOVA test with Bonferroni's correction *, $p<0.05$; **, $p<0.01$; ***, $p<0.0001$.

## Discussion

This study demonstrates that treatment with the synthetic glucocorticoid, dexamethasone, induces transcriptional up-regulation of a-synuclein (AS) in BAC cultured neurons, a progressive PD rat model, corroborating that stress system activation can increase the AS burden and thus, can constitute a "second hit" to study PD pathophysiology. We show 1) downregulation of endogenous AS mRNA in WT cortical neurons only, 2) enhanced expression of AS in WT hippocampal (TX & SDS-soluble) and cortical (SDS-soluble) neurons in the absence of transcriptional up-regulation, 3) transcriptional up regulation of rat and human SNCA mRNA in BAC hippocampal and cortical neurons in the absence of protein expression alterations and 4) higher levels of AS secretion in BAC compared to WT neuronal cultures, with no apparent dexamethasone-dependent effect on AS secretion.

### Dexamethasone increases total AS and pS(129)-AS protein levels in absence of SNCA mRNA upregulation in WT hippocampal and cortical neurons, implicating non-genomic effects

Dexamethasone treatment in WT cultured hippocampal and cortical neurons lead to similar or lower levels of rat SNCA mRNA compared to untreated cells, respectively. These results contradict the findings of Park et al., 2013, who showed that dexamethasone treatment of MN9D cells increased a-synuclein mRNA. However, at the protein level, we showed that 1uM DEX leads to a statistically significant increase of AS protein levels both in soluble and less soluble fractions which corroborates previous studies that showed the same effect of 1uM DEX in WT primary hippocampal neurons (Park et al., 2013). However, in WT hippocampal neurons specific DEX doses lead to increase in levels of total AS. This finding suggests that probably non-genomic effects of glucocorticoids are implicated, which is in line with a plethora of studies demonstrating the non-genomic effects of glucocorticoids mediated mainly via membrane bound-GR, activation of the MAPK pathway (Zhang et al., 2013 ; Limbourg et al., 2003), intracellular calcium elevation (Takahashi et al.,2002) and NOS-dependent NO release as a consequence of ATP-induced intracellular calcium flux (Yukawa et al.,2005). Previous studies from our lab (Clough et al., 2009, 2011) showed that the ERK and PI3-K signaling pathways are involved in the induction of SNCA transcription. Altogether, the absence of SNCA mRNA upregulation at the 24h time-point, yet increased AS protein levels at 48h is likely due to mGR stimulation that triggers rapid non-genomic "priming" effects, ultimately paving the way for the slower genomic activities by glucocorticoids (Vemocchi et al., 2013). On the other hand, concerning the WT cortical neurons, it seems DEX initiates transcriptional down-regulation of rat

SNCA mRNA at all doses. Accordingly, at the protein level DEX, particularly at the 10uM dose, leads to a decrease in total AS levels and shows no effects on secretion AS levels. These results show that dexamethasone down-regulates AS in WT cortical neurons, suggesting a region-specific effect.

### Direct genomic effects of dexamethasone on rat and hu-SNCA mRNA transcription and possible region-specific effects between BAC-transgenic hippocampal and cortical neurons

We showed that 1uM and particularly 5uM DEX leads to a significant induction of rat and human SNCA mRNA in both BAC-transgenic cultured hippocampal and cortical neurons. This finding corroborates previous studies that show transactivation of rat SNCA gene promoter upon DEX administration (Park et al., 2013) but also highlights the high susceptibility of SNCA mRNA in our BAC transgenic rat model of PD compared to WT rats. The increased SNCA expression in BAC hippocampal neurons could be due to defective stress signaling in the hippocampus, a major region implicated in stress pathology and where severely impaired neurogenesis has been demonstrated in BAC rats (Kohl et al. 2016). To further elucidate whether GR activation initiates these genomic effects, we plan to demonstrate the specificity of GR implication by co-treating our cultured neurons with GR antagonists and assessing SNCA mRNA levels. We should also add that statistical significance of SNCA mRNA induction was observed only in BAC-hippocampal neurons compared to corticals, that also exhibit the same pattern of DEX-dependent up-regulation of SNCA mRNA, conforms with the proven highest GR expression in the hippocampus and hypothalamus of humans and rats (Ahima et al., 1991, Wang et al., 2013).

### Dexamethasone induces rat and hu-mRNA levels but shows no effect in total AS and Pser(129)-AS protein levels in BAC-transgenic hippocampal and cortical neurons

We utilized a hu-AS BAC-transgenic rat model (BAC rat) that overexpresses human full-length AS which occurs in familial PD, in which the transgenic construct contains all the necessary human regulatory elements surrounding the SNCA gene (Nuber et al., 2013). In this system, 1 uM and 5 uM DEX induced rat and hu-SNCA transcriptional up regulation 24h after treatment, which translates to no observable increase in AS protein levels and pSer(129) a-synuclein protein levels in the soluble fraction 48h after DEX treatment. Concerning the BAC hippocampal neurons, it seems DEX has no effect on AS protein levels in the Triton soluble material, probably due to increased clearance mechanisms, whereas in the SDS soluble material DEX leads to decreased AS protein levels (particularly 10uM dose) accompanied by an increase in pSer(129) a-synuclein levels that needs further investigation. Overall, more BAC subjects are needed in order to draw safe conclusions. Since we did not identify such a potent DEX effect as we observed in WT neurons, we hypothesize that the observed AS protein levels stability could be due to 1) the already existing overexpression of AS that possibly reaches a plateau which makes it difficult to distinguish the

potency of DEX in this BAC model or 2) increased clearance mechanisms that compensate for the increased transcriptional SNCA up-regulation as a mechanism of cellular homeostasis. The significant increase of pSER(129) AS at 5uM DEX in BAC hippocampal neurons is in accordance with Smith et al., where AS expression levels were the same in all experimental conditions, but treatment with $H_2O_2$ induced a significant increase of AS phosphorylation (at the ser 129 residue). Even though we showed a moderate increase of total and pSER(129) AS protein levels, we did not identify high molecular weight species/oligomers of AS on the Western blots. Future studies will need to utilize cell cytotoxicity assays to ensure that this elevation in AS phosphorylation levels is toxic for the cell and also to discriminate the cytotoxic effects of DEX doses per se by employing the LDH assay to measure membrane integrity and the MTT assay to measure cell viability. Since our in vitro model did not recapitulate formations such as oligomers and species, we plan to proceed to in vivo studies of our BAC model and upon chronic mild stress and DEX/corticosteroid administration to assess the non-addressed issues of our ex vivo experiments. We speculate that the in vivo model will further recapitulate PD pathophysiology since it was previously detected an increased phosphorylation of AS in vivo in a chronic corticosteroid mouse model after a-synuclein fibril injections (Butcher 2018). It is worthy to mention that, that the role of microglia cannot be dismissed as microglia GR are specifically implicated in PD neurodegeneration (Ros bernal et al., 2011) so future studies will include DEX effects in neuronal-glial co-cultures, too.

## Dexamethasone had no apparent effect in secretion of AS neither in WT nor in BAC neuronal cultures

Utilizing an ultra sensitive ELISA (Emmanouilidou et al., 2011) for the detection of total AS in cell lysates and cultured condition media of WT and BAC transgenic hippocampal neurons, we show that BAC-transgenic neurons' conditioned media secrete higher levels of AS compared to WT neurons, in accordance with previous studies (Reyers et al.,2005). Another interesting finding is that treatment with 1 and 5uM DEX, in WT hippocampal neurons, endogenous levels of total AS are higher than the levels of secreted AS, compared to untreated cells. One speculation could be that, given the increased levels of insoluble pSER-129 AS with 5uM DEX in BAC neurons, which is proposed to facilitate the initiation of toxic oligomeric AS, the insoluble AS forms that either will result in LB formation (that will be assessed in vivo) or are released into the extracellular environment- species which cannot be detected by our ELISA. Another hypothesis, proposed by Panettieri et al., 2019, could be that glucocorticoids' effects in lipid rafts affect the posttranslational modifications of AS and consequently, its secretion. Previous studies suggest a raft-associated post-translational modification of AS indicating both phosphorylation and O-linked

glycosylation of α-synuclein (Pronin et al., 2000; Ellis et al., 2001; Nakamura et al., 2001; Shimura et al., 2001). One study demonstrated that the A30P mutation primarily eliminates AS binding to lipid rafts (Fortin et al., 2004). In addition, findings confirmed the colocalization and crosstalk between mGR and caveolin-1 (the main component of caveolae, a subset of lipid rafts) which leads to ERK phosphorylation (Watson et al., 2012); a significant pathway that upregulates SNCA transcription, according to our lab's previous work (Clough et al., 2011). However, the apparent decrease in the levels of secreted AS at 1 and 5uM DEX doses may result from the increased endogenous AS levels and may not necessarily be ascribed to less secretion.

## Conclusions

Chronic stress seems to exacerbate PD by direct or indirect regulation of SNCA gene. We showed a DEX-driven up-regulation of the SNCA gene and increased AS and insoluble pSER(129)-AS protein levels in primary neurons of hu-AS BAC rats, indicating that glucocorticoid receptor activation alone can increase the AS burden and thus, could potentially trigger or exacerbate PD pathogenesis in vivo. Although the exact components of this mechanism are not yet discovered, further in vitro and in vivo studies will hopefully elucidate this stress-related molecular pathway that either triggers or exaggerates PD pathophysiology and provide new avenues of research into therapeutic interventions.


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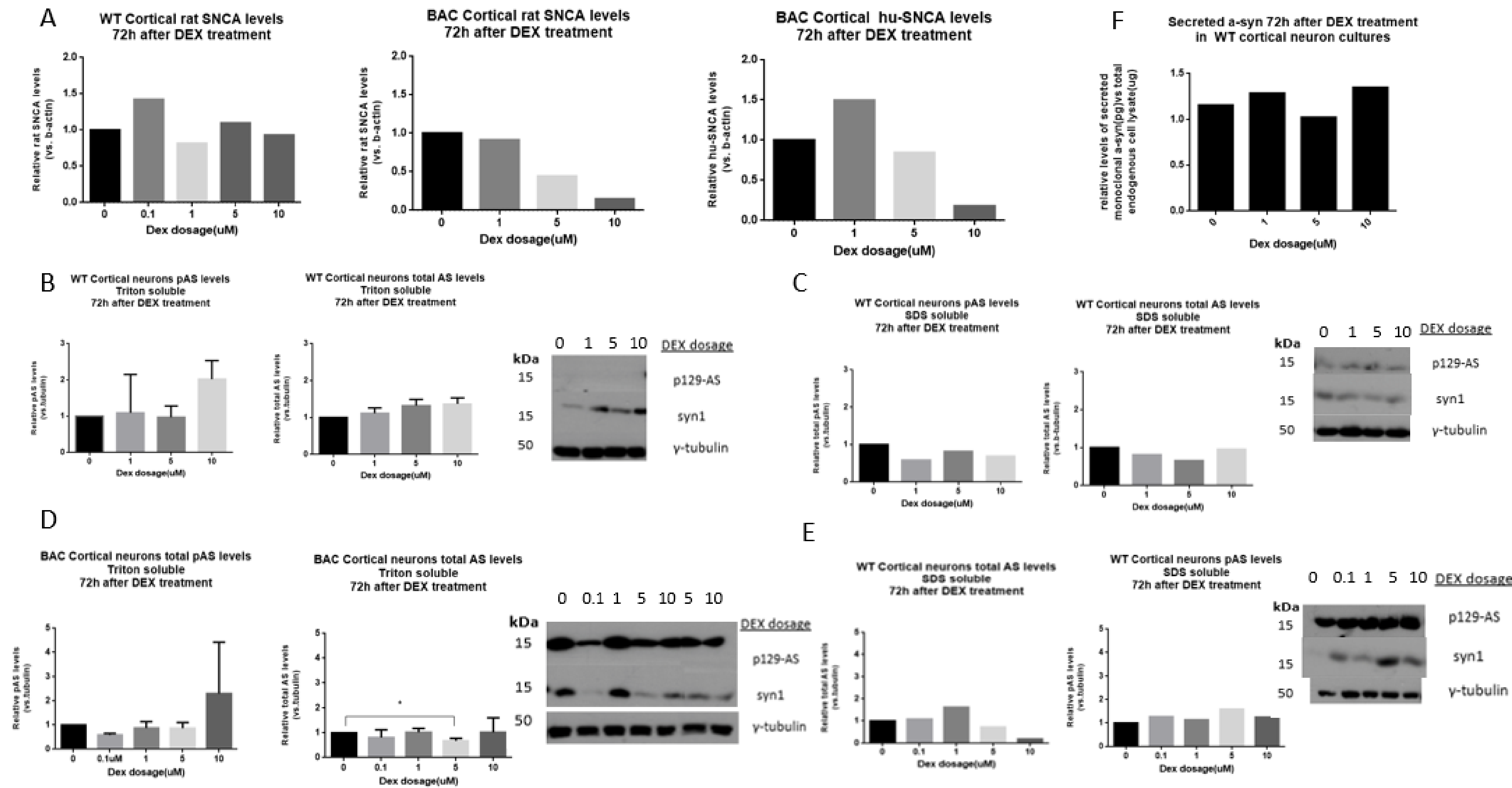
# Supplementary DATA




**Supplementary Figure 1. Transcription, Expression and Secretion effects of DEX treatment in AS 72h after treatment.** WT and BAC-transgenic cortical neuronal cultures were prepared from embryonic day 17 (E17) rats. The neurons were treated at 6 DIV with DEX doses (0, 0.1, 1, 5 10uM). The primary neuronal cultures were maintained in 2.5% enriched medium (see also Table 1, Methods). A, At 72h post-treatment rat and human SNCA mRNA levels were assessed with RT-PCR, for WT and BAC cortical neurons. One experiment was performed in triplicate for WT and BAC SNCA mRNA levels. Results are presented as means S.E. (*n*=3, *n*=3, respectively). *B-E*, At 72h post-treatment total a-synuclein (AS) and pSER(129)-a-synuclein (pAS) protein levels (both Triton-X and SDS soluble) were assessed with Western Blotting. γ-tubulin, loading control. B,D, for WT cortical total a-synuclein (AS) and pSER(129)-a-synuclein (pAS) protein levels (Triton-X soluble) two independent experiment performed in triplicate, in WT and BAC cortical neurons, respectively. Results are presented as means S.E. (*n*=6, *n*=6, respectively). C,E, for WT cortical total a-synuclein (AS) and pSER(129)-a-synuclein (pAS) protein levels (SDS soluble) one experiment was performed in triplicate, in WT and BAC cortical neurons, respectively. Results are presented as means S.E. (*n*=3, *n*=3, respectively). F, At 72h post-treatment, cultured condition media of BAC and WT neurons were collected to measure secreted monoclonal AS with ELISA. Secreted AS protein levels are expressed as picograms(pg) and normalized with total protein levels of cell lysates (Triton-X soluble) expressed in micrograms(ug) per condition. A, up-regulation of rat SNCA mRNA in 0.1 and 5uM DEX in WT cortical neurons, but not statistical significant. Down regulation of rat SNCA mRNA in all DEX doses in BAC cortical neurons, but not statistical significant. Up-regulation of hu-SNCA mRNA in 1uM DEX in BAC cortical neurons but not statistical significant. B,C, In WT cortical neurons, DEX had no effect in pAS levels (Triton-

X and SDS soluble) but there is a slight increase in 5uM DEX in Triton-X (but not statistical significant) and absence of up-regulation of AS in SDS soluble protein fraction. D, efficient down-regulation of AS and pAS levels (Triton-X soluble) in 1 and 5uM, respectively, that led to statistical significant decrease in 5uM DEX ($p<0.05$), in BAC cortical neurons. E, there is a trend of increased pAS and AS levels (SDS soluble) in 1 and 5uM DEX, respectively, but not statistical significant, in BAC cortical neurons. F, there is a trend of increased secretion of monoclonal AS in 1 and 10uM DEX but not statistical significant, in WT cultured cortical condition media. Statistical analysis was performed via an unpaired *t* test . *, *p* 0.05; **, *p* 0.01; ***, *p* 0.0001.