Identification of DOT1L Inhibitor in a Screen for Factors that Promote Dopaminergic Neuron Survival

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Keywords: Parkinson's disease, DOT1L, DOT1L inhibition, SGC0946, dopaminergic neuron, high throughput screen, neural progenitor cells, human pluripotent stem cell (hPSC)

Abstract

Parkinson's disease (PD) is a common neurodegenerative disorder characterized by the progressive loss of dopaminergic (DA) neurons in the substantia nigra region of the midbrain. Diagnostic criteria for PD require that at least two of three motor signs are observed: tremor, rigidity, and/or bradykinesia. The most common and effective treatment for PD is Levodopa (L-DOPA) which is readily converted to DA and has been the primary treatment since the 1960's. Dopamine agonists have also been developed but are less effective than L-DOPA. Although the lack of a model system to study PD has hampered efforts to identify treatments, diverse screening strategies have been proposed for identification of new pharmaceutical candidates. Here, we describe a pilot screen to identify candidate molecules from a bioactive compound library, that might increase formation, maintenance and/or survival of DA neurons *in vitro.* The screen used a previously characterized reporter construct consisting of the luciferase gene inserted downstream of the endogenous tyrosine hydroxylase (TH) gene and neurons differentiated from human pluripotent stem cells for 18 days. The reporter mimics expression of TH and includes a secreted luciferase whose activity can be measured non-invasively over multiple timepoints. Screening of the bioactive compound library resulted in the identification of a single molecule, SGC0946, that is an inhibitor of DOT1L (Disruptor Of Telomeric silencing 1-Like) which encodes a widely-conserved histone H3K79 methyltransferase that is able to both activate and repress gene transcription. Our results indicate that SGC0946 increased reporter luciferase activity with a single treatment at 8-hours post-plating being equivalent to continuous treatment. Moreover, data suggested that the total number of neurons differentiated in the assays was comparable from experiment to experiment under different SGC0946 treatments over time. In contrast, data suggested that the survival and/or maintenance of DA neurons might be specifically enhanced by SGC0946 treatment. These results confirm other reports that indicate inhibition of DOT1L may play an important role in maintenance and survival of neural progenitor cells (NPCs) and their lineage-specific differentiation.

Introduction

Parkinson's disease (PD) is a common neurodegenerative disorder that affects almost one million people in the United States and more than 6 million internationally (Collaborators, 2018). Although the symptoms of PD vary among patients, a central hallmark is the progressive loss of movement control associated with the progressive loss of dopaminergic (DA) neurons of the substantia nigra of the midbrain (Albin et al., 1995). Intensive study over the years has identified

numerous environmental and genetic risk factors that lead to PD in a subset of cases; however, the cause of the majority of PD remains poorly understood (Bloem et al., 2021).

One of the major limitations that hinders research in PD is the lack of access to affected human DA neurons. Thus, the development of human pluripotent cells including both human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) has demonstrated the potential to provide a reliable source to obtain human DA neurons and study the disease. hESCs are pluripotent cells that have been derived from the inner cell mass of an early-stage embryo (Thomson et al., 1998) while iPSCs are generated from adult somatic cells, usually skin fibroblast cells, that are induced to pluripotency via a combination of four transcription factors (Takahashi and Yamanaka, 2006, Takahashi et al., 2007). Advances over the last decade or so, in defining differentiation conditions have made it possible to directly drive differentiation of human pluripotent cells to DA neurons and use these derived neurons to model PD in a dish (Yang et al., 2008, Swistowski et al., 2010, Gonzalez et al., 2013, Chambers et al., 2009, Zhang et al., 2014, Kriks et al., 2011, Kedariti et al., 2022, Stern et al., 2022, Singh et al., 2017, Barbuti et al., 2020). Multiple studies from different research laboratories have also shown that these derived neurons not only have cellular and biochemical characteristics very similar to the neurons developed in the human brain but also are functional when transplanted into animal models (Byers et al., 2015, Doi et al., 2014, Grealish et al., 2014, Kriks et al., 2011, Byers et al., 2011, Hoban et al., 2020) Thus, studies over the last decade plus have demonstrated that human pluripotent stem cells can provide a reliable source for generating DA neurons for *in vitro* disease modeling and have the potential to contribute to regenerative treatment options for the disease (Behl et al., 2022).

Developing treatments for PD traditionally has focused predominantly on relief of symptoms, as there is currently no effective mean(s) to restore DA neurons that are lost in PD patients. One potentially feasible option is to restore these neurons in patients via transplantation of *in vitro* derived DA neurons. Another approach is to screen for chemicals that have a protective effect directly on DA neurons. Pharmaceutical chemicals that may improve survival of DA neurons have multiple uses in PD related research and future applications: The immediate application of such chemicals is to enhance the production of a larger number of DA neurons more efficiently in *in vitro* derivation of DA neurons. Further, pharmaceutical chemicals would also provide a window into PD to enable mapping of biological function(s) of pathways that are compromised in PD. Finally, such chemicals might also slow or halt loss of DA neurons *in situ* during progression of PD.

As indicated above, DA neurons differentiated *in vitro* have the potential to provide high quality human midbrain DA neurons that are fully functional both in *in vitro* and *in vivo* assays and applications. Yet, it is clear that current differentiation methods are still time consuming and the number of derived neurons is often limited greatly by variation in the differentiation efficiencies from one experimental replicate to another. Applications that use derived DA neurons either for *in vitro* cellular modeling or for future cell replacement therapies would benefit greatly from the improvement of the differentiation methods and/or development of methods that distinguish DA neuron survival in a complex cell mixture. Here, we review the characterization of an hESC reporter line that has a secreted luciferase reporter gene inserted into the endogenous tyrosine hydroxylase (TH) gene in order to provide a rapid and efficient method to measure differentiation and survival of DA neurons in complex differentiation cultures (Bademci et al., 2012) Then, we present results of a pilot screening study, based on a well-characterized, commercially-available Tocris chemical library, that was aimed at identifying compounds that may improve the survival of DA neurons during direct differentiation *in vitro,* in adherence to culture plates and/or in post-differentiation survival.

Materials and Methods

The Tocris library: We chose to screen the Tocriscreen 2.0 library for potential factors that would act to promote differentiation, adherence and/or survival of DA neurons. This library was obtained from Bio-Techne Corporation (Minneapolis, MN) in a 96-well format with each chemical dissolved in 10mM DMSO with an indicated shelf-life of at least 6 months. The 1280 compounds within the Tocriscreen 2.0 library cover such research areas as cancer, immunology, neuroscience and stem cells. Pathways include the G-protein coupled receptors (GPCRs), kinases, enzymes and enzyme-linked receptors, chemicals that target pathways of cell biology and ion channels. For screening, day 18 DA neuron progenitors were plated and cultured for 10 days with DMSO only (control) or treated with chemicals during the first 48 hr (single treatment) or 8 days (continuous treatment). TH expression was detected using the luciferase assay and normalized to the treatment with DMSO only. Results were graphed with X axis representing the reading of single chemical treatment and Y axis representing continuous treatment for 8 days. Luciferase results were confirmed by Q-PCR of the luciferase and TH RNA.

Directed dopaminergic differentiation and quantification of TH using luciferase assay. Pluripotent human stem cells (hESCs) were differentiated towards a dopamingeric neuron cell fate following the floor-plate induction method(Zhang et al., 2014). Cell supernatants were collected every day for the first 20 days of neuronal differentiation and every other day afterwards to ensure that the protocol produced DA neurons. Luminescent reactions were performed by mixing the supernatants with 10× reaction buffer consist of 300 mM Tris-HCl pH 8.0, 40 mM NaCl, 1% Triton X-100 and 200 μM substrate Coelenterazine (NanoLight; Flagstaff, AZ). Luminescent signal was measured using a Synergy H1 Plate Reader (BioTek; Santa Clara, CA). Background signal level was measured using blank medium with reaction buffer and subtracted from experimental data.

Drug screening assays. hESCs were differentiated towards dopamingeric neurons for 18 days as described above. One parameter that directly affected the yield of DA neurons in the final culture in our previous study was cell density (Cui et al., 2016). To identify the optimal cell density for DA neuron differentiation and test performance of the TH luciferase reporter assay, we replated differentiated neural cells at different densities and followed the DA neuron growth for two weeks. When cells were plated at densities higher than 400 K cells per well, restraint by the surface growth area greatly limited neuron attachment and growth resulting in massive cell detachment within several days. In contrast, fewer than 50 K cells per well led to very poor cell attachment and a low total number of DA neurons. Within the optimal range, each well produced 10^3 to 10^4 units of luminescence signal when the culture matured and signals showed a linear correlation with cell numbers on Day 40, suggesting the cell growth was not restricted during the culturing period. The growth test was also performed in the 384-well format and exhibited a similar result with optimal cell densities of 20 K to 100 K cells per well. These results indicated that the reporter is suitable for used as luciferase based quantitative assays within the optimal range of cells per well. Given these results, on Day 18, neurons were dissociated by incubating with Accutase (Innovative Cell Technologies; San Diego, CA) for 5 minutes. Dissociated cells were counted and replated to 96-well plates coated with poly-L-ornithine/laminin/fibronectin at a density of 3×10^5 cells/cm^2 and allowed to recover for one week in neurobasal medium containing B-27 supplement, BDNF, GDNF, cAMP, TGFb, and ascorbic acid. Drug treatment was performed as previously described for neurotoxins (Grealish et al., 2014). Fleshly prepared Tocris solutions were diluted with the basal medium and added to each well to reach the desired final concentration. Assays were done with multiple wells of 4 replicates for each condition. Whole supernatants of the same well were collected before drug treatment and 48 hours post treatment for quantification using luciferase assay as described above. Viability of TH+ cells was calculated as the percentage of

147 luminescent signal intensity post treatment compared to that before treatment of the same well.
148
149 **RNA extraction and quantitative PCR analysis.** Total RNA was extracted using RNeasy mini
150 kit (Qiagen, Hilden, Germany). For first-strand cDNA synthesis, 2 µg of total RNA was reverse
151 transcribed with oligo-dT primers and SuperScript III reverse transcriptase (Life Technologies,
152 Carlsbad, CA). Quantitative PCR reactions were run using Power SYBR PCR mix (Life Technologies,
153 Carlsbad, CA) and detected by ABI 7300 Real-Time PCR System. Primers used for PCR are listed in
154 **Table 1.** Relative quantity for each sample and gene was normalized and calculated based on the
155 ∆Ct values using GAPDH as the control.
156
157 **Results**
158 In order to screen for chemicals that might enhance DA differentiation and/or survival, we used a
159 genetic reporter system that we previously constructed and demonstrated was able to monitor
160 differentiation efficiency and compare DA survival under different conditions (Cui et al., 2016). We
161 note here that the reporter contains an insertion of a luciferase reporter gene into the endogenous
162 TH locus, the gene that encodes the enzyme governing the rate-limiting step in dopamine
163 production (Cui et al., 2016). The luciferase reporter was shown to enable rapid non-invasive
164 quantification of dopaminergic neurons in cell culture throughout the entire differentiation process
165 (Cui et al., 2016). Moreover, luciferase was shown to be under the same endogenous regulation
166 of the TH gene demonstrating that the cellular assay is effective in assessing neuron response to
167 different cytotoxic chemicals and able to be scaled for high throughput applications suggesting
168 feasibility of use as a quantitative cellular model for toxin evaluation and drug discovery.
169
170 We differentiated the reporter-containing pluripotent hESCs, previously described (Cui et al.,
171 2016) to midbrain DA neurons using an established floor-plate induction protocol with defined
172 combinations of growth factors (Kriks et al., 2011, Zhang et al., 2014). The engineered cells had
173 previously been shown to differentiate to TH+ DA neurons with similar robustness as the parental
174 cell line in both a 96-well and 384-well format, with stable expression of TH observed starting from
175 Day 11 of induction and continuing to increase throughout the following culturing, which correlated
176 with the timing of midbrain DA neuron growth and maturation (Cui et al., 2016).
177
178 To screen the Tocriscreen 2.0 library, day 18 DA neuron progenitors were plated and cultured for
179 10 days with DMSO only (control) or treated with chemicals during the first 48 hr (single treatment)
180 or 8 days (continuous treatment) as diagrammed **(Figure 1A).** TH expression was detected using
181 the luciferase assay and normalized to the treatment with DMSO only. Results were graphed with
182 X axis representing the reading of single chemical treatment and Y axis representing continuous
183 treatment for 8 days. We identified a single chemical component of the Tocriscreen 2.0 library,
184 SGC0946, that resulted in a significant increase (>175% increase with either single or continual
185 treatment relative to the DMSO Only control (**Figure 1B).** We noted that multiple other chemical
186 components of the Tocris library demonstrated smaller effects while the majority of chemicals
187 demonstrated a reduction in luciferase activity relative to the DMSO Only control.
188
189 We then compared TH expression as assessed by luciferase assay to expression values obtained
190 by qPCR of the luciferase gene insert (TH-Luc) or the endogenous TH gene (TH11-12). We
191 observed that the luciferase readings of Day 18 plated DA neuron progenitors (following 10-days
192 differentiation) corresponded to the TH expression by qPCR of both TH-Luc and TH11-12, as
193 shown (**Figure 2A-B).**
194
195 Finally, we compared gene expression of a marker of total neurons (TUBB3 (BetaIII Tubulin)) to
196 markers that are reported to be specific to DA neurons (Dopamine Transporter (DAT), GIRK 2
197 and NURR1). As shown in **Figure 3A,** there is no difference between control and treated total

neurons following either single or continuous treatment with SGC0946. In contrast, markers specific to DA neurons (DAT, GIRK2 and to a lesser extent, NURR1) demonstrated an increase in expression with treatment with SGC0946 relative to DMSO Only control as shown in Figure 3B. A longer treatment of SGC0946 increases the expression of DAT, while the expression of GIRK2 and NURR1 can only benefit from a shorter period of SGC0946 treatment during the first 24 or 48 hours of DA neuron differentiation.

Discussion

Over the last decade or more, diverse screening strategies have been proposed for identification of new pharmaceutical candidates to treat PD (Barbuti et al., 2020, Hideshima et al., 2022, Leah et al., 2021, Schikora et al., 2021, Aldewachi et al., 2021, d'Amora and Giordani, 2018, Dawson et al., 2019, Smith et al., 2017, Stewart, 2014, Lavecchia and Giovanni, 2013). Strategies include high-content screening to generate single-cell gene-corrected patient-derived pluripotent stem cell clones useful for further exploration of excess alpha-synuclein with familial PD mutations and identification of perturbed pathways, two-step screening method to identify inhibitors of α-synuclein aggregation, high throughput screens of mitochondrial, neuron or neurite morphology, RPPA (Reverse Phase Protein Arrays) and antibody-based proteomic approaches, and strategies that employ organoids, model organisms and virtual (*in silico)* screening (Barbuti et al., 2020, Hideshima et al., 2022, Leah et al., 2021, Schikora et al., 2021, Aldewachi et al., 2021, d'Amora and Giordani, 2018, Dawson et al., 2019, Smith et al., 2017, Stewart, 2014, Lavecchia and Giovanni, 2013). Given the array of assays that are available for high throughput screening, it is hoped that the use of multiple assays might lead to positive intersections of the most promising treatments for PD, understanding of underlying causes and identification of growth factors and pathways that might be modulated.

Here, we report the use of a human pluripotent stem cell-based system, for discovery of neural toxins and protective factors, in a pilot screen for factors that might enhance differentiation, survival and/or maintenance of DA neurons differentiated *in vitro.* In this pilot screen, we identified SGC0946, an inhibitor of DOT1L (Disruptor Of Telomeric silencing 1-Like) which encodes a widely-conserved histone H3K79 methyltransferase that is able to both activate and repress gene transcription. Our results indicate that SGC0946 increased reporter luciferase activity with a single treatment at 8-hours post-plating being equivalent to continuous treatment. Moreover, data suggested that the total number of neurons differentiated in the assays was comparable from experiment to experiment under different SGC0946 treatments over time. In contrast, data suggested that the survival and/or maintenance of DA neurons might be specifically enhanced by SGC0946 treatment. These results provide evidence in support of other reports that indicate inhibition of DOT1L may play an important role in maintenance and survival of neural progenitor cells (NPCs) and their lineage-specific differentiation (Ferrari et al., 2020, Gray de Cristoforis et al., 2020).

Previously we performed a ChIP-sequencing study and characterized dopamine neuron transcriptional and epigenetic programs including the global binding profiles of H3K27ac, H3K4me1, and 5-hydroxymethylcytosine [5hmC]) at four different stages of development(Xia et al., 2017). We demonstrated the dynamic pattern of epigenetic modification during DA neuron differentiation and maturation. The discovery of the linkage between DOT1L and DA neurons in our compound screen here suggests that H3K79me might also play a key regulatory role during DA development.

As noted in previous primary publications and in a comprehensive review, DOT1-Like (DOT1L) is the sole methyltransferase of histone H3K79 (Wong et al., 2015, Ferrari et al., 2020, Gray de Cristoforis et al., 2020, Wille and Sridharan, 2022). DOT1L can orchestrate mono- di- or tri-

methylation that is correlated with actively transcribing genes (mono- and di-methylation) or repression (tri-methylation). Moreover, DOT1L has been recognized as a promising epigenetic target for solid tumors and has been extensively studied in MLL (multi-lineage leukemia) genomic rearrangements and sporadic fusion proteins that may drive errors in the localization of H3K79 methylation and subsequent oncogenesis (Alexandrova et al., 2022). More recently, studies have demonstrated that loss of function of DOT1L is accompanied by perturbations in development of somatic lineages and in reprogramming *in vitro* (Wille and Sridharan, 2022). Thus, the evidence suggests that DOT1L is broadly required for differentiation, that reduced DOT1L activity is concomitant with increased developmental potential and that loss of DOT1L activity results in more upregulated than downregulated genes. DOT1L also participates in various epigenetic networks that are both cell type and developmental stage specific and may play an important role in maintenance and survival of neural progenitor cells (NPCs) and their lineage-specific differentiation (Ferrari et al., 2020, Gray de Cristoforis et al., 2020). Nonetheless, to our knowledge, this is the first association of DOT1L activity and formation, differentiation and/or maintenance of DA neurons. Clearly, further exploration of this association is merited.

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

JC, JoC, and RRP all contributed to the research described in this manuscript: JC, JoC and RRP each contributed to the experimental design, analysis of data and interpretation of the results. JC prepared the manuscript figures; JoC outlined the methods and materials section and RRP prepared the final manuscript for publication.

Funding

This work was supported by private funds from the Mallett Family (San Francisco, CA) and the Weissman family (Palo Alto, CA). McLaughlin Research Institute also supported this work.

Acknowledgments

We thank members of the Reijo Pera laboratory and the McLaughlin Research Institute, past and present, for their helpful comments and review of this work.

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404 **Table 1. Primers Used in This Study.**

Th11-12 Forward	TGGAGTTCGGGCTGTGTAAG
Th11-12 Reverse	AGCTCCTGAGCTTGTCCTTG
Th-Luc Forward	CAGCCCTACCAAGACCAGAC
Th-Luc Reverse	CTCCCAGATGGTGAACAGGT
TUBB3 Forward	GGCCTTTGGACATCTCTTCA
TUBB3 Reverse	ATACTCCTCACGCACCTTGC
GIRK2 Forward	CGCTGATCATTAGCCATGAA
GIRK2 Reverse	ATGGGGTGCTGGTCTCATAG
NURR1 Forward	GGCGAACCCTGACTATCAAA
NURR1 Reverse	CTGGGTTGGACCTGTATGCT
DAT Forward	TGAGCTCTTCACGCTCTTCA
DAT Reverse	CACCATAGAACCAGGCCACT
GAPDH Forward	AAGGTGAAGGTCGGAGTCAACG
GAPDH Reverse	TGGACTCCACGACGTACTCAGC

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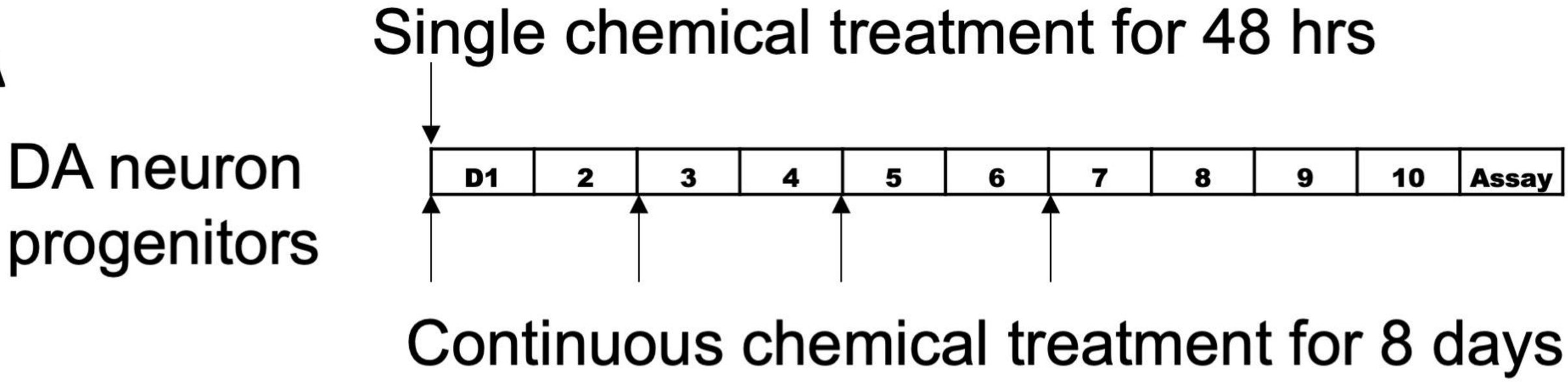
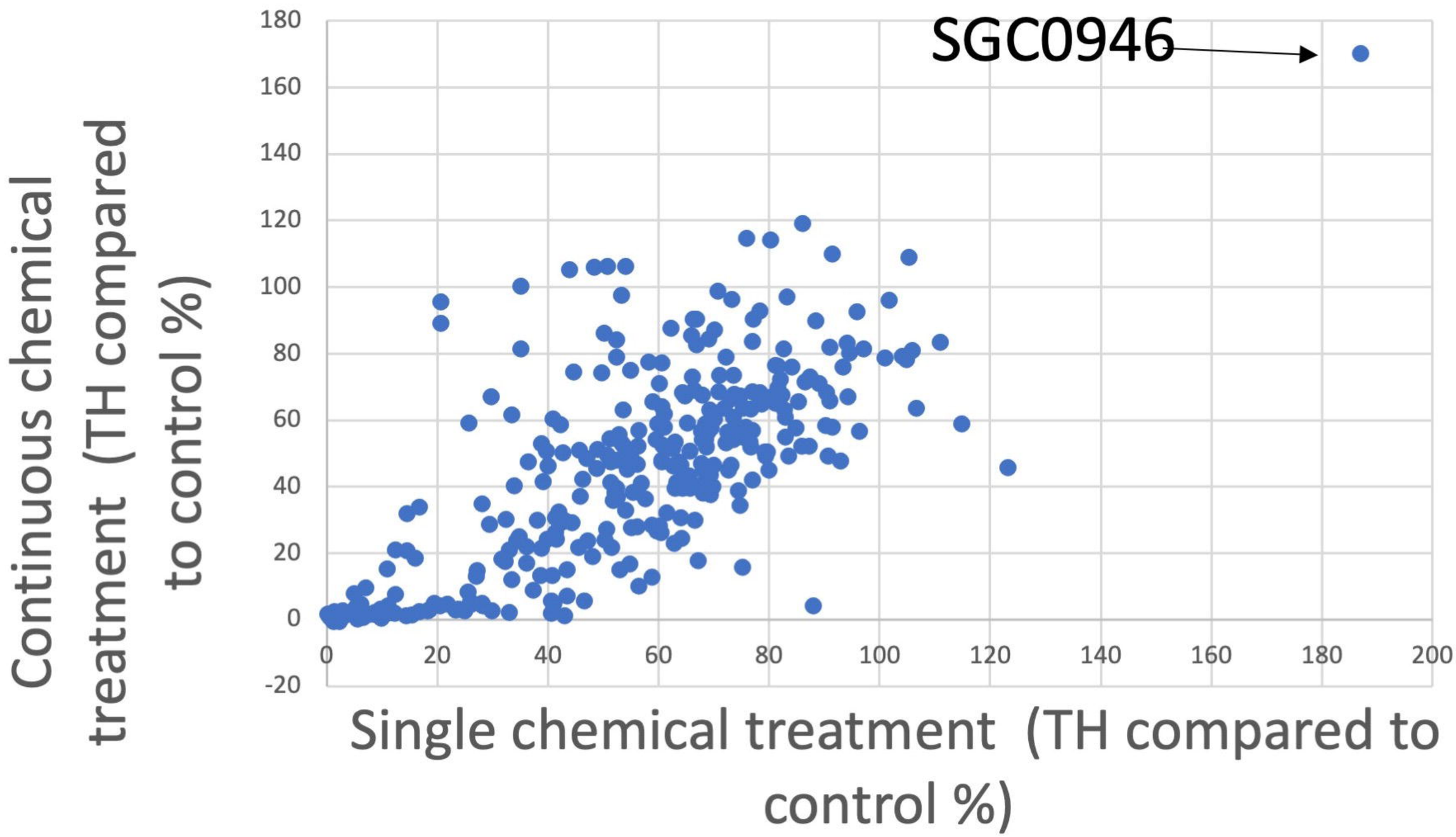
408 **Figure Legends**
409
410 **Figure 1. Screen of Tocriscreen 2 chemical library. (A)** Experimental design. Day 18 DA
411 neuron progenitors were plated and cultured for 10 days with DMSO only (Control) or treated
412 with chemicals during the first 48 hr (single treatment) or 8 days (continuous treatment).
413 Luciferase readings were done at the end of the 10-day differentiation. **(B)** Screen results. TH
414 expression was detected using the luciferase assay and normalized to the treatment with DMSO
415 only. X axis represents the reading of single chemical treatment and Y axis represents
416 continuous treatment for 8 days.
417
418 **Figure 2. Tyrosine hydroxylase (TH) reporter activity. (A)** TH expression by luciferase
419 assay. Day 18 DA neuron progenitors were cultured for 10 days with DMSO only (Control) or
420 treated with SGC0946 for the first 24 hr, 48 hr and 8 days. Luciferase readings were done at the
421 end of the 10-day differentiation. **(B)** TH expression by qPCR of the luciferase gene (TH-Luc) or
422 the endogenous TH gene (TH11-12).
423
424 **Figure 3. Gene Expression by qPCR. (A)** SGC0946 treatment does not increase the number
425 of total neurons as measured by expression of TUBB3 (BetaIII Tubulin). **(B)** SGC0946 treatment
426 potentially increases the number of dopaminergic neurons as measured by the expression of
427 TH and Dopamine Transporter (DAT). Data suggests that a short treatment is preferred for the
428 midbrain DA neurons as measured by the midbrain DA neuron marker GIRK 2. NURR1 is a
429 widely expressed marker on neurons, microglia and other cell types.
430

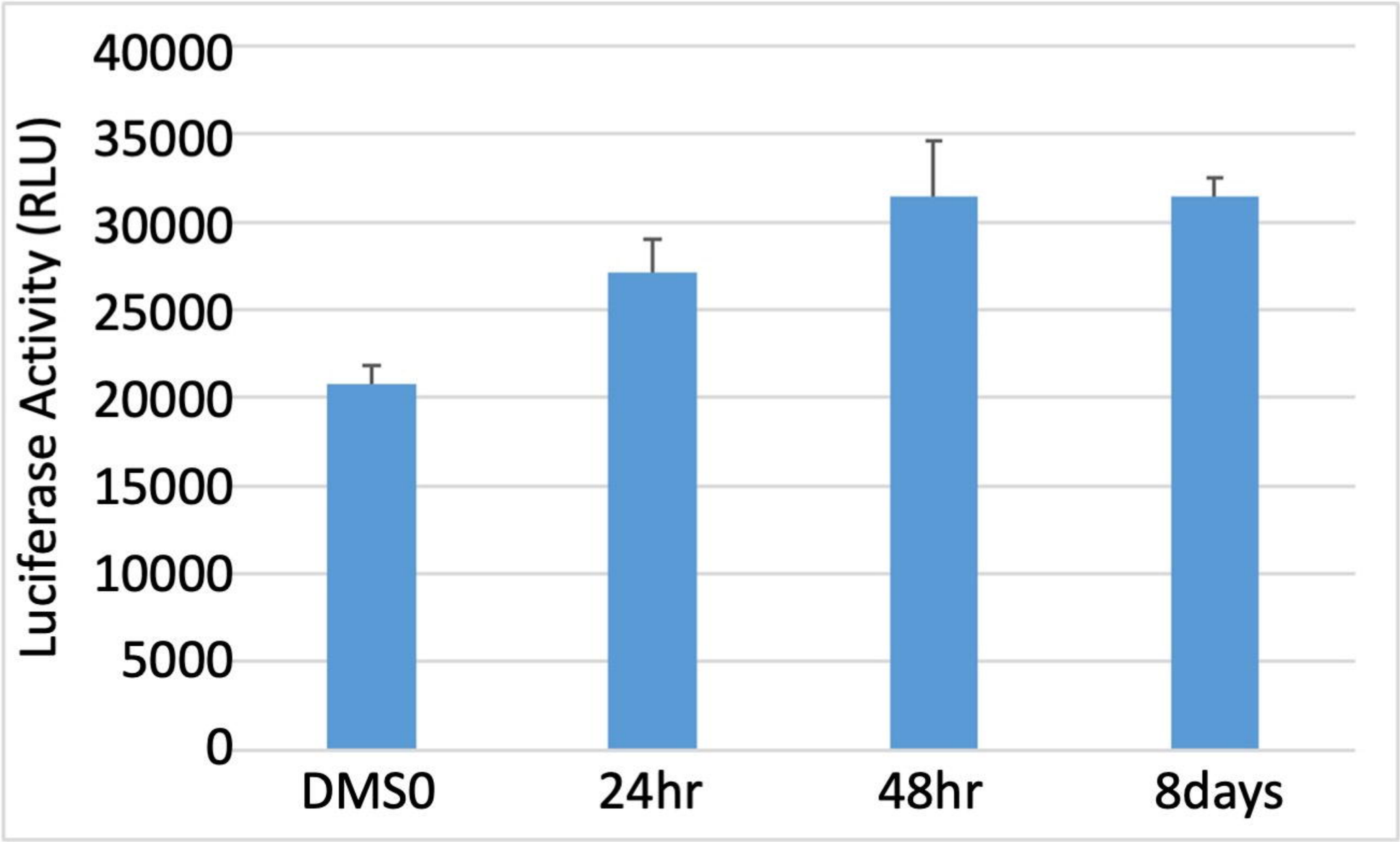
A


Single chemical treatment for 48 hrs
DA neuron progenitors
D1
2
3
4
5
6
7
8
9
10
Assay
Continuous chemical treatment for 8 days

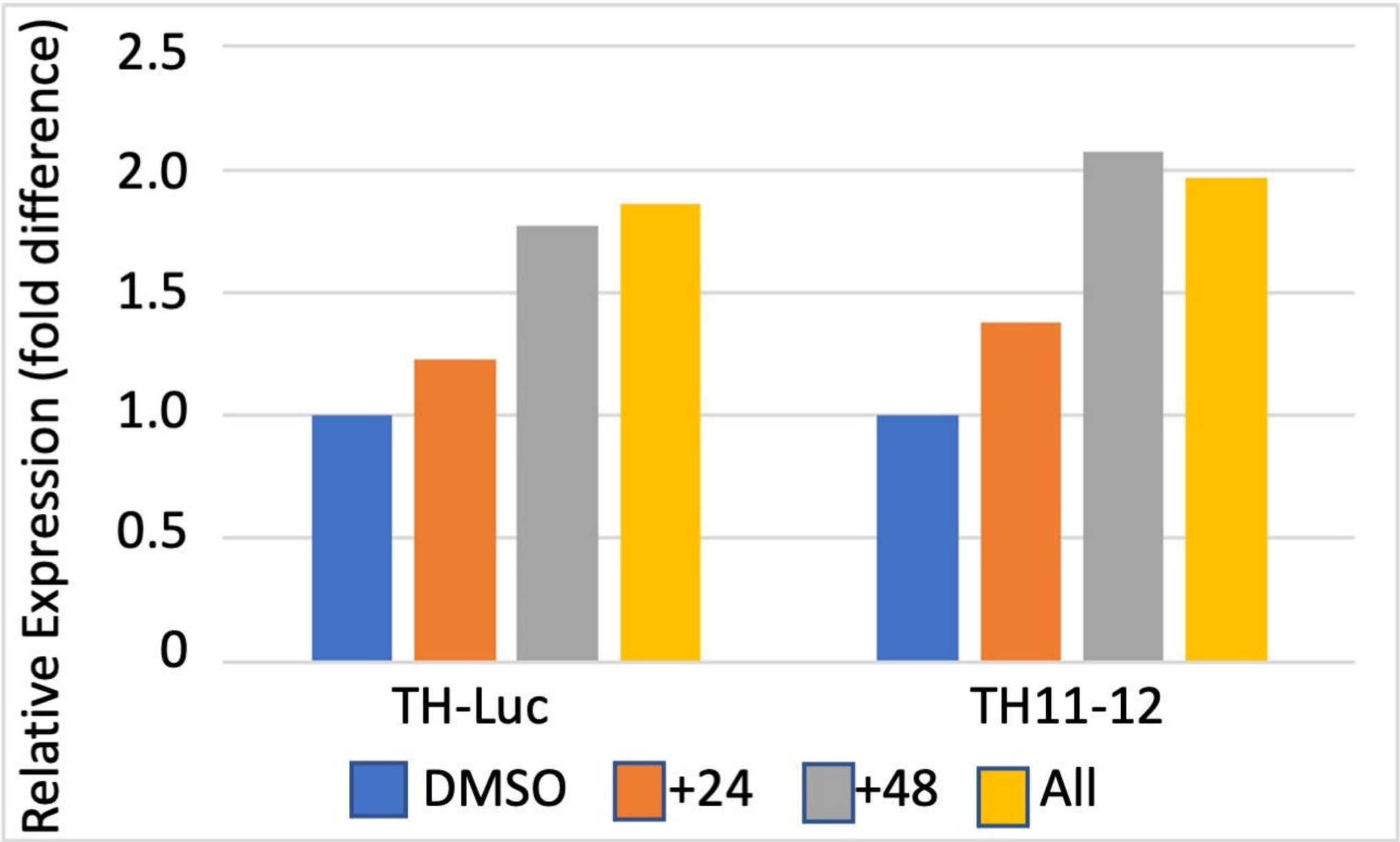
B


SGC0946
Continuous chemical treatment (TH compared to control %)
Single chemical treatment (TH compared to control %)
180
160
140
120
100
80
60
40
20
0
-20
0
20
40
60
80
100
120
140
160
180
200

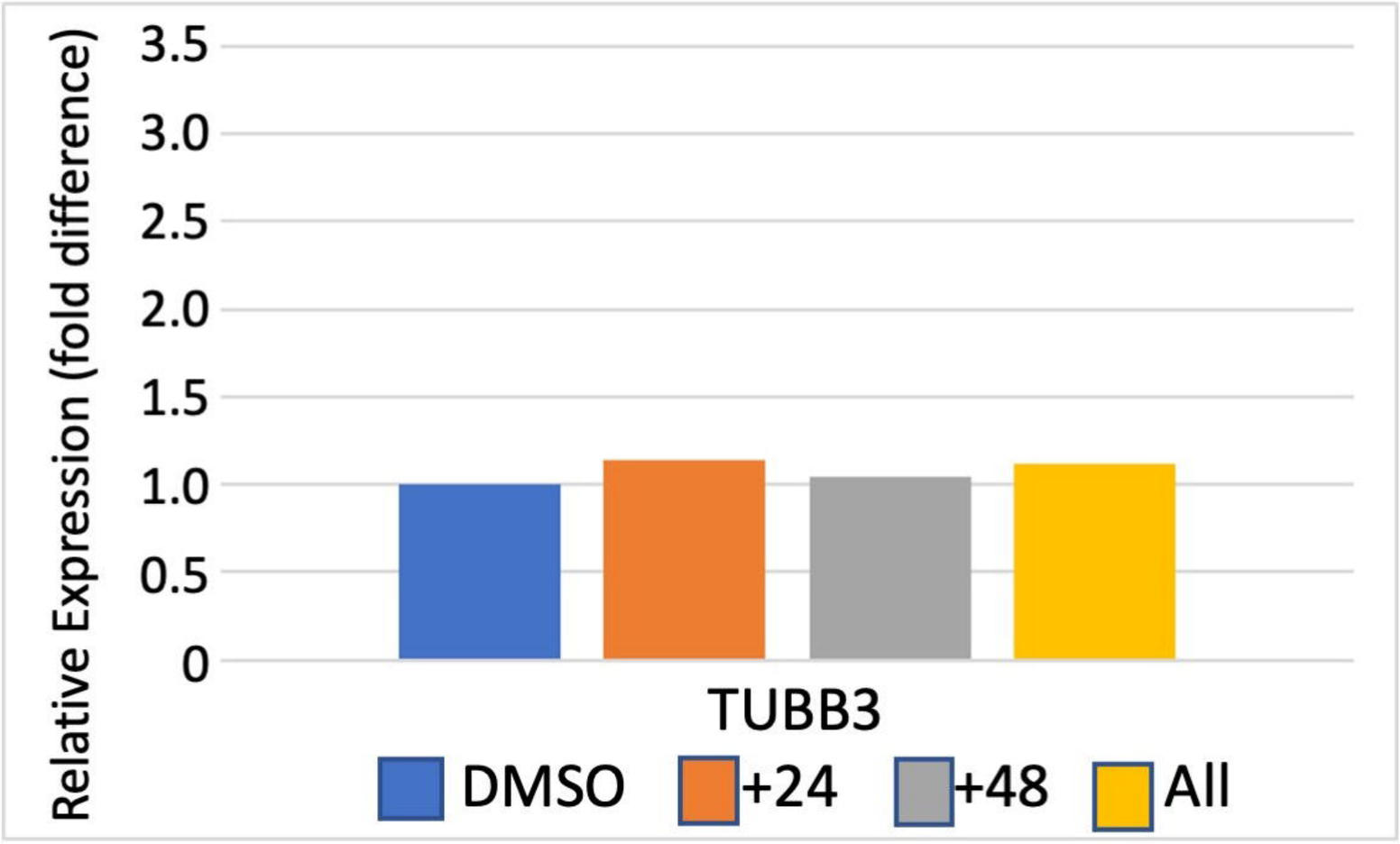
A


Luciferase Activity (RLU)
40000
35000
30000
25000
20000
15000
10000
5000
0
DMS0
24hr
48hr
8days

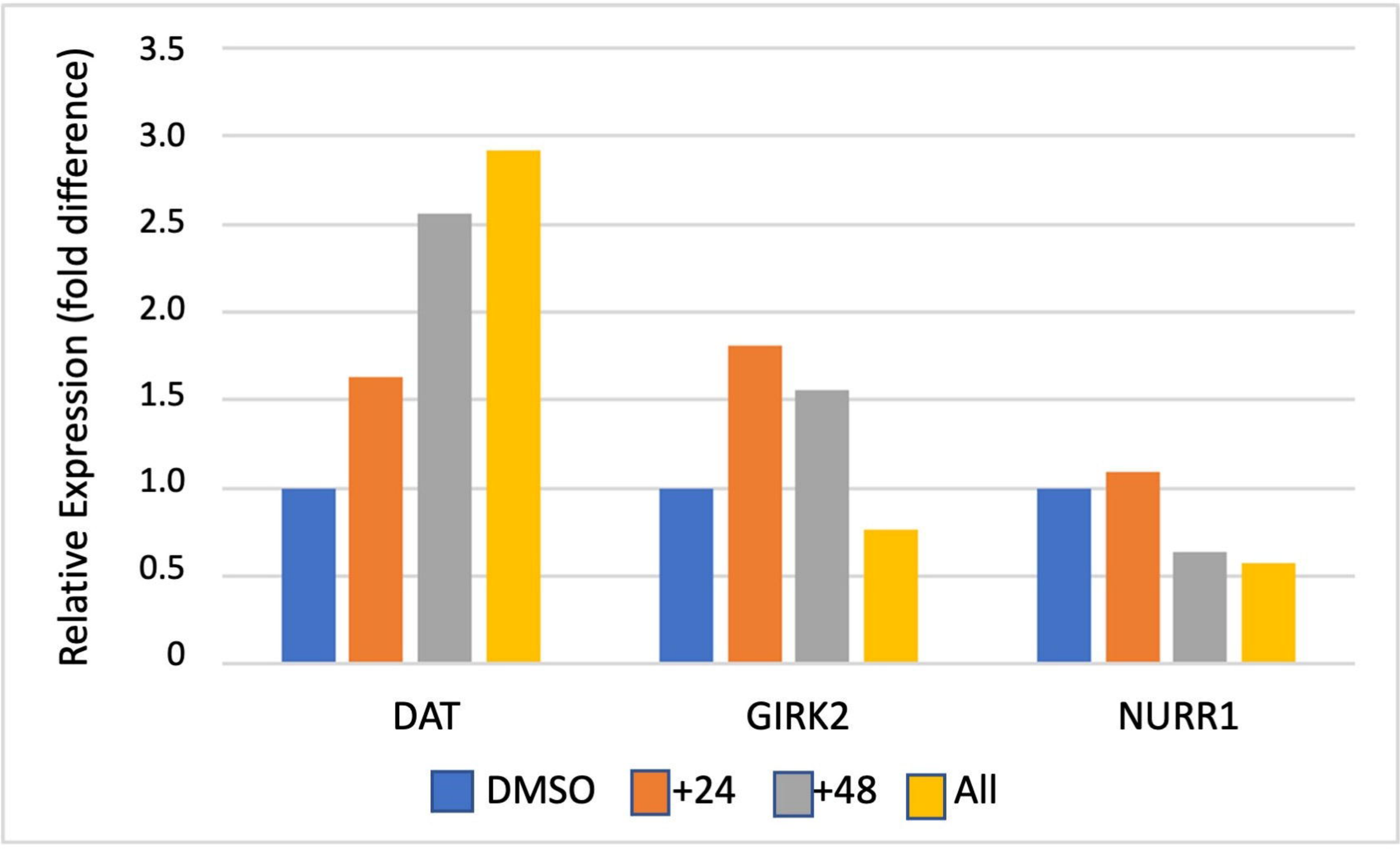
B


Relative Expression (fold difference)
2.5
2.0
1.5
1.0
0.5
0
TH-Luc
TH11-12
DMSO
+24
+48
All

A


Relative Expression (fold difference)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
TUBB3
DMSO
+24
+48
All

B


Relative Expression (fold difference)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
DAT
GIRK2
NURR1
DMSO
+24
+48
All