Alterations in proliferation of neuronal stem cells in Attention-Deficit/Hyperactivity Disorder and Wnt modulation by methylphenidate

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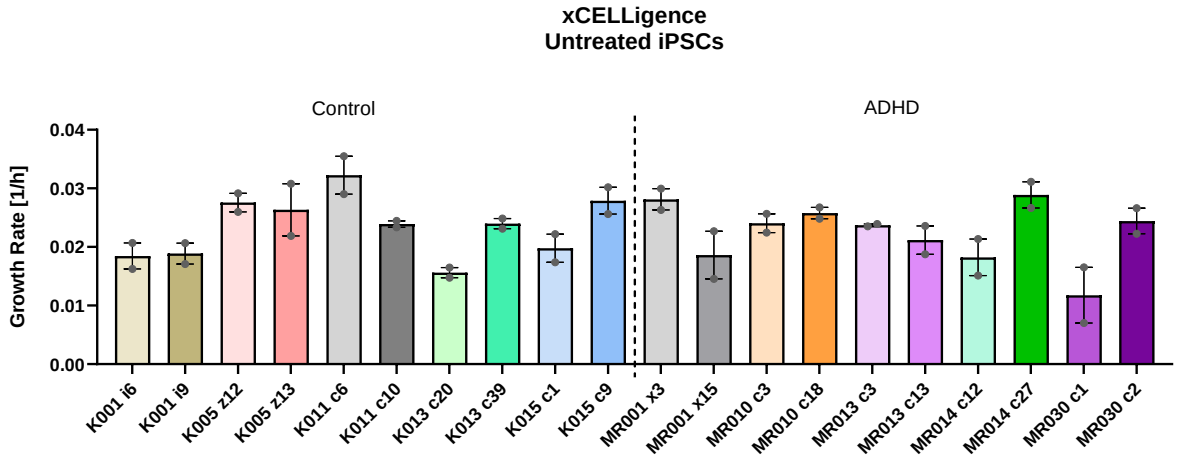
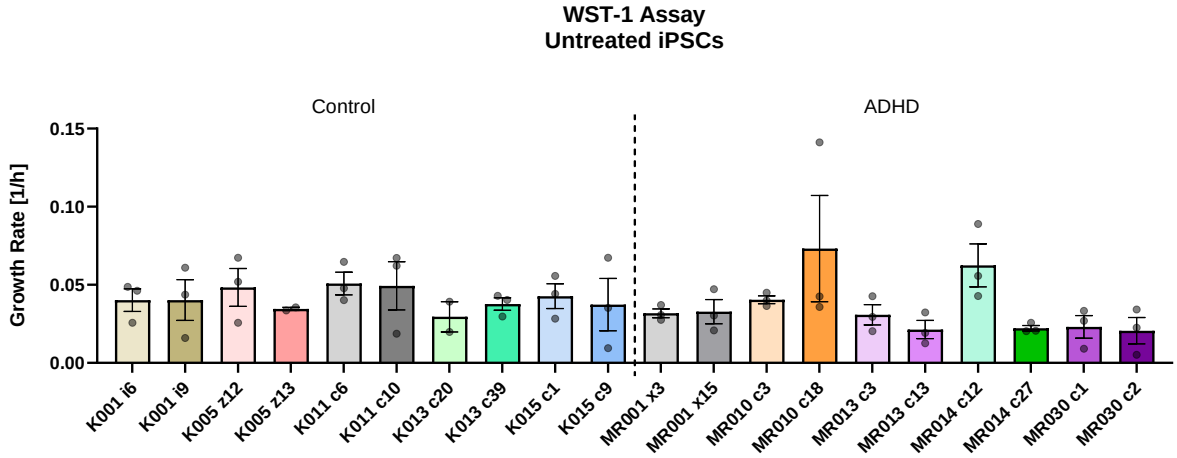
This file contains Supplementary Figures 1 – 6 and Supplementary Tables 1, 2 and 3 from the manuscript from Yde Ohki et al. Supplementary Tables 4 and 5 are individually attached.

Supplementary Information

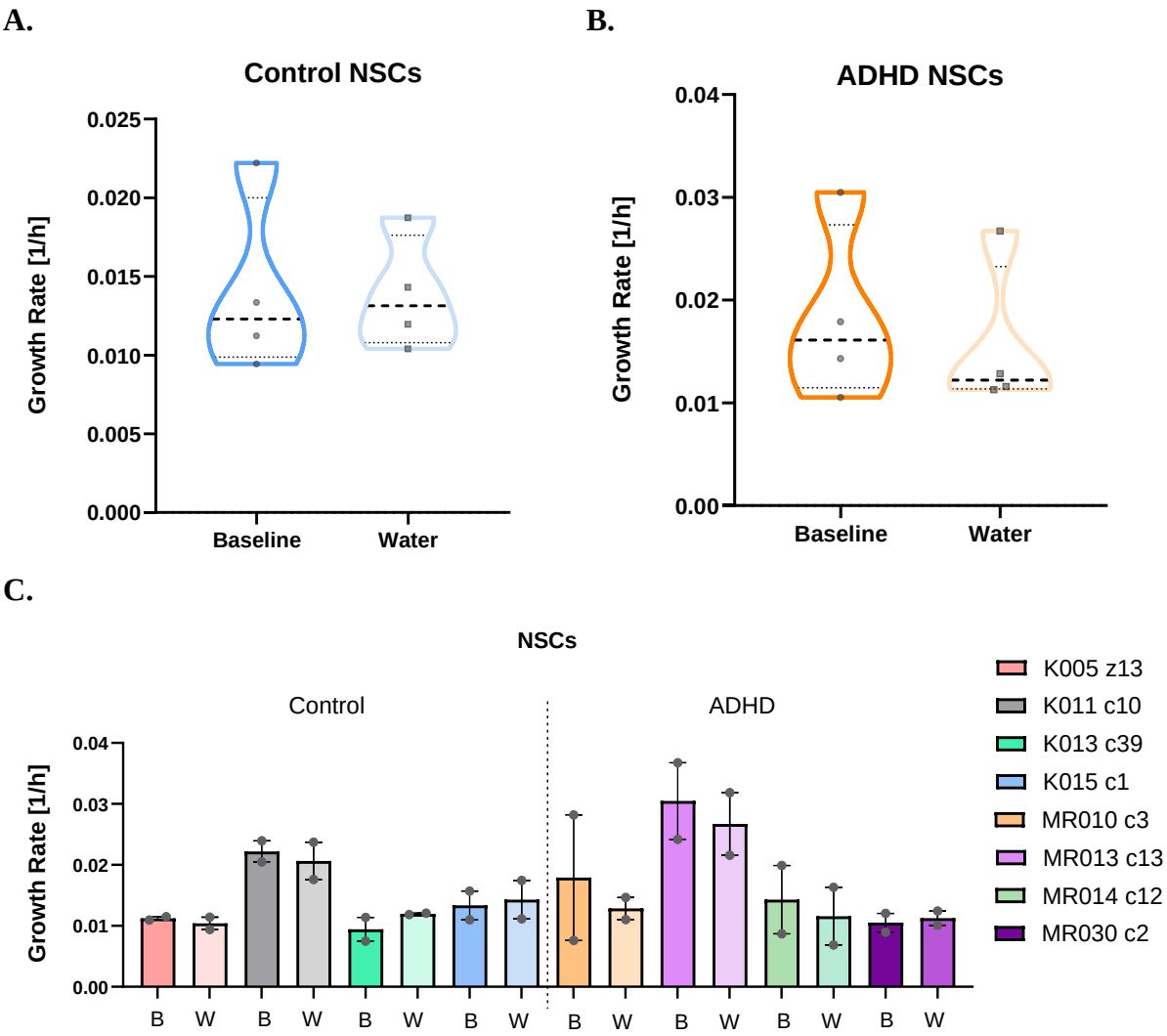
A.



B.

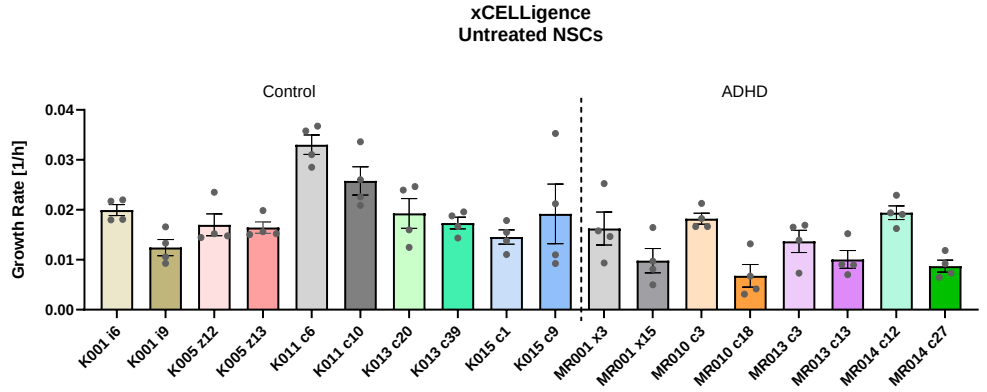


Supplementary Fig. 1: Growth rates from all iPSC lines included in this study at baseline. Growth rates derived from xCELLigence experiments (A) and WST-1 assays (B) are shown in the graphs. Mean ± SEM for each cell line is depicted, while each dot represents one technical replicate. At least 2 technical replicates were considered to calculate individual means in this analysis.



Supplementary Fig. 2: Growth rates from NSC lines cultured as baseline or with water as vehicle treatment. Growth rates derived from xCELLigence experiments in control (A) and ADHD (B) NSCs are shown in the graphs. For both groups, no differences are seen between the conditions (Mann-Whitney, n.s.). C) Growth rates from individual cell lines in these two conditions. B= Baseline condition; W = Water condition. Mean ± SEM for each cell line is depicted, while each dot represents one technical replicate. N=4 Control individuals (1 clone each) and 4 ADHD patients (1 clone each) were analyzed in 2 technical replicates.

A.



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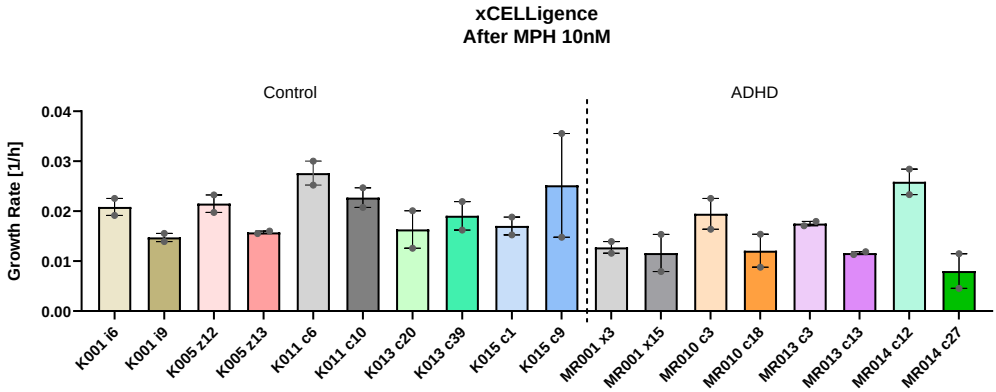
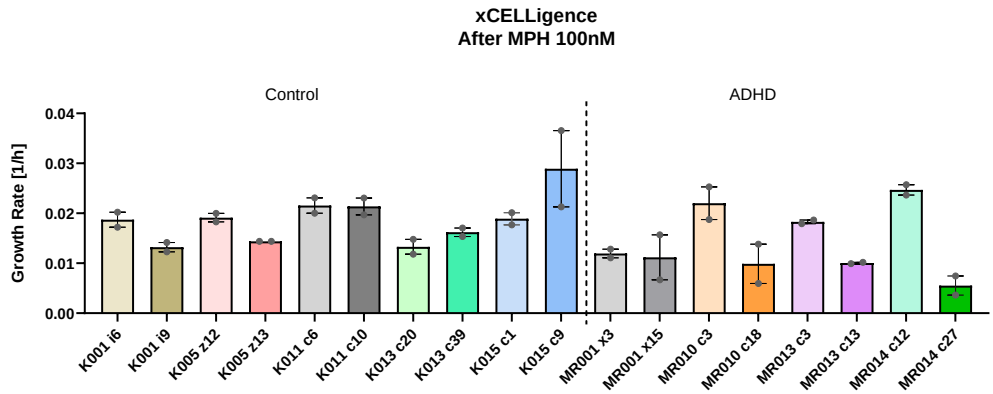
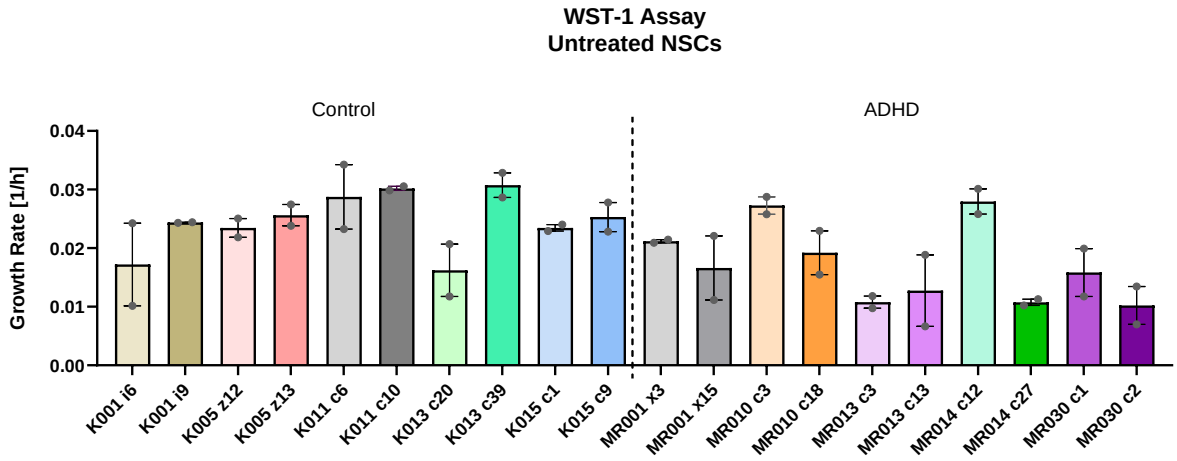


C.



Supplementary Fig. 3: Growth rates of all the NSC lines analyzed in xCELLigence. The graphs depict growth rates for individual NSC lines at baseline (A) and after chronic treatment with MPH 10 nM (B) and 100 nM (C). For the vehicle condition, four experiments per cell line were considered. Mean ± SEM for each cell line is depicted, while each dot represents one technical replicate. N=2 technical replicates were performed for each cell line.

A.



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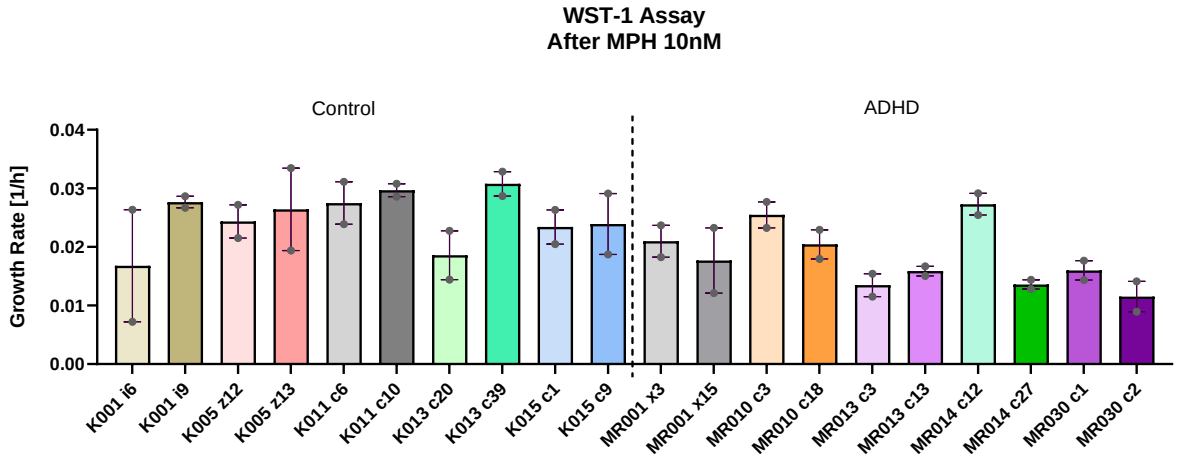
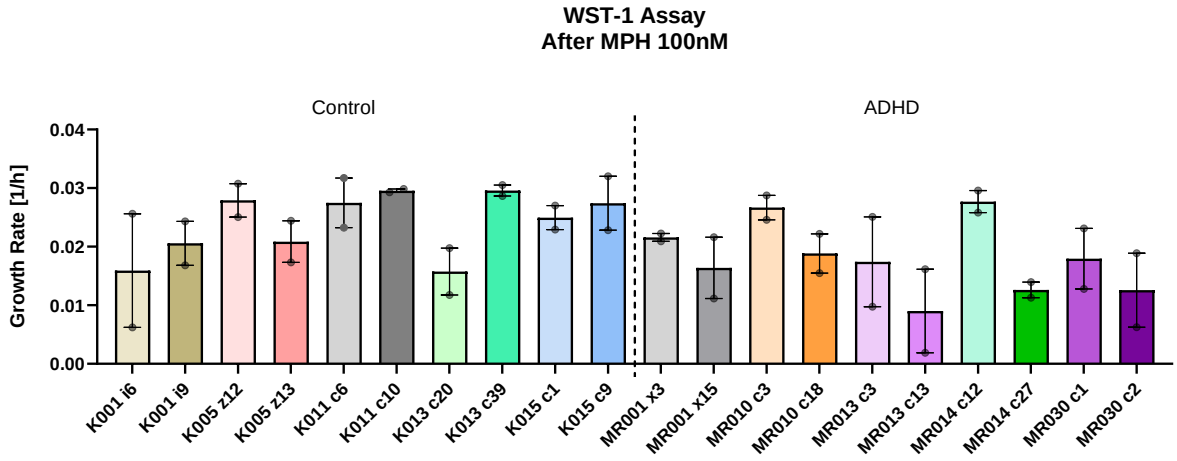
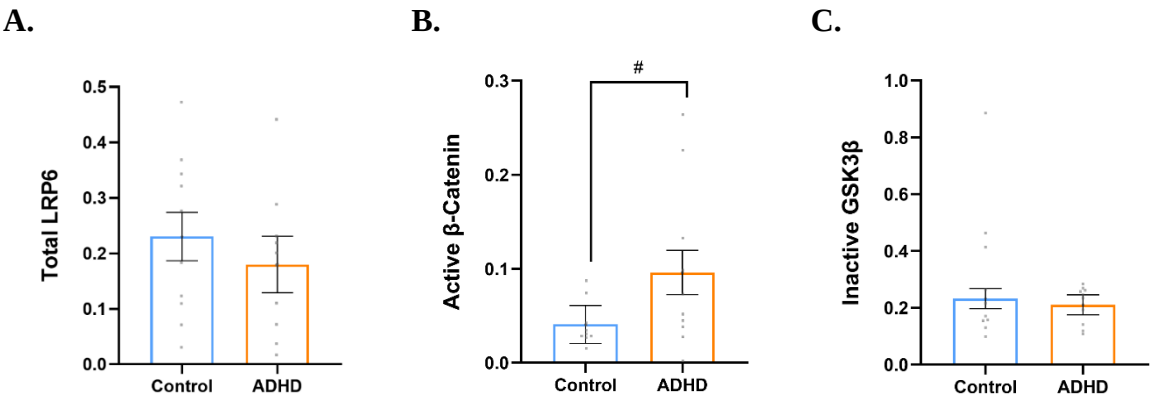


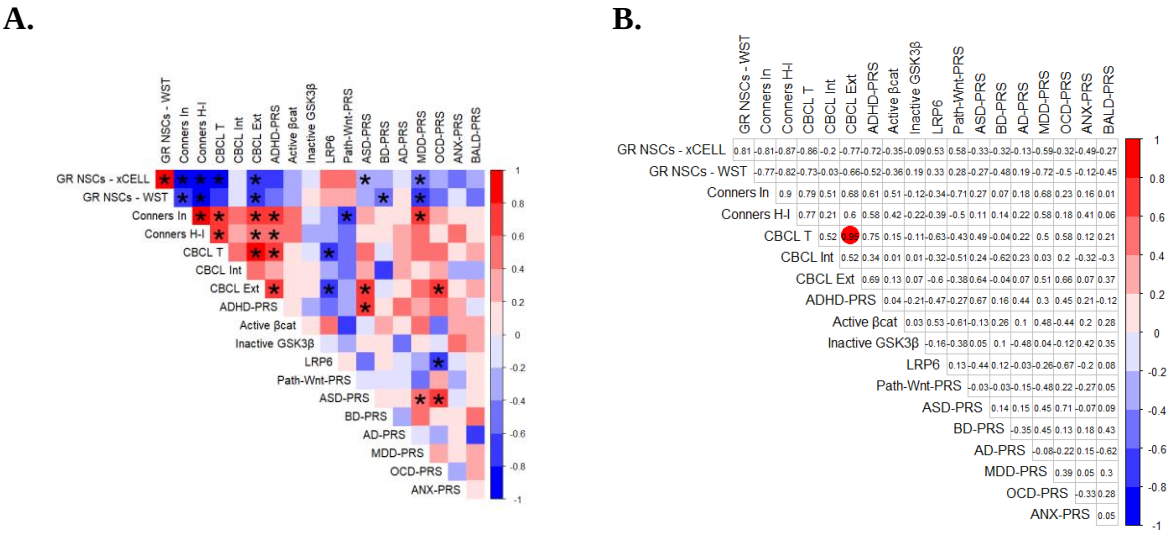
C.



Supplementary Fig. 4: Growth rates of all the NSC lines analyzed in WST-1 assays. The graphs depict growth rates for individual NSC lines at baseline (A) and after chronic treatment with MPH 10nM (B) and 100nM (C). Mean ± SEM for each cell line is depicted, while each dot represents one technical replicate. N=2 technical replicates were performed for each cell line.



Supplementary Fig. 5: Protein expression of Wnt-related proteins between control and ADHD NSCs at baseline after 7-day culture: Total LRP6 (A), active β-catenin (B) and inactive GSK3β (C). No statistical differences were seen for any of the proteins. A trend toward significance was observed for active β-catenin ($^{\#}p$ = 0.068). Mean ± SEM (model-adjusted, standard *lmer* for total LRP6 and active β-catenin; robust *lmer* for inactive GSK3β) is depicted, while each dot represents the averaged raw data from 2 experiments per cell line. N=2 technical replicates were performed for each cell line.



Supplementary Fig. 6: Correlations between ADHD-related behavioral scores, Wnt-PRS, PRS for ADHD and other disorders and *in vitro* findings when results from two iPSC clones were averaged. A) Uncorrected pairwise comparisons after averaging *in vitro* findings of two clones derived from the same donor. As in Fig. 5A, Spearman's correlations were conducted in this analysis while colors code the strength and direction of each correlation. Asterisks indicate statistically significant correlations without any corrections applied ($p < 0.05$). B) Correlation coefficients after Bonferroni corrections are shown. After a conservative Bonferroni's correction for 171 unique pair combinations, 1 pair survived the correction (0.05/171, in red, $p < 0.05$): CBCL T *versus* CBCL Ext. The red color indicate the positive correlation whereas 0.95 represents the correlation coefficient. Abbreviations: GR NSCs – xCELL: Growth rates of NSCs from xCELLigence assays; GR NSCs – WST: Growth rates of NSCs from WST-1 assays; Conners In = Inattention scores from Conners' Rating Scales; Conners H-I: Hyperactivity/Impulsivity scores from Conners' Rating Scales; CBCL T = Total Scores from CBCL; CBCL Int = Internalizing scores from CBCL; CBCL Ext = Externalizing scores from CBCL; Active βcat = Protein expression of active β-catenin after a 7-day cell culture; Inactive GSK3β = Protein expression of inactive GSK3β after a 7-day cell culture; LRP6 = Protein expression of LRP6 after a 7-day cell culture; ADHD-PRS = Polygenic Risk Scores for Attention-Deficit Hyperactivity Disorder; Path-Wnt-PRS = Pathway-PRS specific to Wnt signaling; ASD-PRS = Polygenic Risk Scores for Autism Spectrum Disorder; BD-PRS = Polygenic Risk Scores for Bipolar Disorder; AD-PRS = Polygenic Risk Scores for Alzheimer's Disease; MDD-PRS = Polygenic Risk Scores for Major Depressive Disorder; OCD-PRS = Polygenic Risk Scores for Obsessive-Compulsive Disorder; ANX-PRS = Polygenic Risk Scores for Anxiety Disorder. Genetic variables and clinical scores were also present in a previous publication[27].

Supplementary Table 1: Demographic and clinical information about subjects analyzed in this study.

Cell line	Clones analyzed in this study	Cell type of origin	Diagnosis	Age	Sex	ADHD-PRS (z-score)	IQ	Conners		CBCL		
								I	H/I	Total	Ext	Int
K001	i6 & i9	Keratinocytes	Healthy control	15	Male	0.46	118	57	50	54	51	55
K005	z12 & z13	Keratinocytes	Healthy control	16	Male	-0.52	113	53	48	48	45	53
K011	c6 & c10	Keratinocytes	Healthy control	16	Male	-1.03	124	39	48	43	34	51
K013	c20 & c20	PBMCs	Healthy control	9	Female	-0.08	127	43	47	52	53	53
K015	c1 & c9	PBMCs	Healthy control	13	Male	-1.66	127	30	38	39	40	49
MR001	x3 & x15	Keratinocytes	ADHD	15	Male	-0.67	121	73	69	63	60	61
MR010	c3 & c18	Keratinocytes	ADHD	9	Male	1.63	133	73	69	63	60	61
MR013	c3 & c13	PBMCs	ADHD	16	Male	0.29	109	73	73	53	50	49
MR014	c12 & c27	PBMCs	ADHD	13	Male	0.45	98	73	58	59	57	57
MR030	c1 & c2	PBMCs	ADHD	9	Female	1.13	143	66	69	68	74	50

Individual ADHD-PRS were calculated using Demontis et al. 2023[31] and a threshold of p=0.05 was adopted. T-values from Conners' Rating Scales and Child Behavior Checklist (CBCL) scores are represented. Abbreviations: I = Inattention; H/I = Hyperactivity/Impulsivity; Total = Total scale scores; Ext = Externalizing scale scores; Int = Internalizing scale scores.

Supplementary Table 2: Primary and secondary antibodies for Western Blot

Type of Antibody	Primary Antibodies	Product	Company	Species	Dilution	Size
Primary Antibody	Anti-β-Catenin non-phosphorylated, active	D13A1	Cell Signaling Technology	Rabbit	1: 1000	92 kDa
	Anti-GSK3β	ab32391	Abcam	Rabbit	1: 5000	46 kDa
	Anti-GSK3β Phospho S9	ab75814	Abcam	Rabbit	1: 5000	47 kDa
	Anti-LRP6	ab134146	Abcam	Rabbit	1: 500	180 kDa
	Anti-GAPDH loading control	ab8245	Abcam	Mouse	1: 2000	40 kDa
Secondary Antibody	Goat Anti-Rabbit IgG (HRP)	ab7090	Abcam	Goat	1:10000	N/A
	Donkey Anti-Mouse IgG(HRP)	ab7061	Abcam	Donkey	1:10000	N/A

105 **Supplementary Table 3: Statistics from the *posthoc* tests applied in this study.**

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Experiment	Figure	Comparison	Est.	SE	t- or z-ratio	df	p-value
NSC Proliferation after MPH treatment using xCELLigence assays	2B	ADHD Vehicle *versus* Control Vehicle	-0.37	0.13	-2.89	14.00	**0.012**
		ADHD MPH 10 nM *versus* Control MPH 10 nM	-0.29	0.13	-2.23	14.00	**0.043**
		ADHD MPH 100 nM *versus* Control MPH 100 nM	-0.27	0.13	-2.10	14.00	*0.055*
NSC Proliferation after MPH treatment using WST-1 assays	2C	ADHD Vehicle *versus* Control Vehicle	-0.30	0.11	-2.80	9.54	**0.020**
		ADHD MPH 10 nM *versus* Control MPH 10 nM	-0.27	0.11	-2.57	9.54	**0.029**
		ADHD MPH 100 nM *versus* Control MPH 100 nM	-0.24	0.11	-2.29	9.54	**0.046**
NSC Proliferation after MPH and/or DKK1 treatments using xCELLigence assays	3B	ADHD Vehicle *versus* Control Vehicle	-0.36	0.17	-2.16	12.60	*0.051*
		ADHD MPH 10 nM *versus* Control MPH 10 nM	-0.28	0.17	-1.65	12.60	0.124
		ADHD DKK1 *versus* Control DKK1	-0.29	0.17	0.11	12.60	0.110
		ADHD DKK1 + MPH *versus* Control DKK1 + MPH	-0.33	0.17	-1.96	12.60	*0.073*

107 Statistically significant p-values ($p < 0.05$) are highlighted in **bold**, while trends toward significance ($0.05 < p <$
108 0.075) are written in *italic*.

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Supplementary Table 4: Pair of all correlations considering clones individually (N=20), as well as their respective correlation coefficients and p-values.

See file attached as supplementary material.

Supplementary Table 5: Pair of all correlations after averaging results from clones (N=10), as well as their respective correlation coefficients and p-values.

See file attached as supplementary material.