Supplemental Material

Fingerprinting of neurotoxic compounds using a mouse embryonic stem cell dual luminescence reporter assay

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Table S1

Classification of compounds in three classes based on clinical evidence for neurotoxicity

Non-neurotoxic (no evidence for DNT/NT)	Possibly (developmental) neurotoxic	Well-established (developmental) neurotoxic
Saccharin	Panobinostat*	Valproic acid[e]
Ibuprofen	Entinostat*	Methylmercury[f]
Omeprazole	Belinostat*	PBDE-99[g]
Nicotinic acid	Methoxyacetic acid*	PCB-153[h]
Uric acid	Phenylmercuric acetate**	Arsenic trioxide[i]
D-mannitol	4-chloromercuric benzoic acid**	Trimethyltin chloride[l]
Propranolol	Mercury bromide**	Chlorpromazine[m]
G-CSF	Thimerosal**	
Erythropoietin	Triadimefon***	
Neuregulin	Cyproconazole***	
Oxytocin	Geldanamycin***	
Sildenafil	Abiraterone***	
Rivaroxaban	IFN-beta***	
	Nintedanib [a]	
	Amiodarone [b]	
	Sulfadiazine [c]	
	Imatinib [d]	
	Gefitinib[n]	

*Belonging to HDACi family or similar mode of action as valproic acid, a known DNT toxicant (Jentink et al. 2010);

** Organo-mercury compounds, similar mode of action as methylmercury, known DNT/NT toxicant (Grandjean and Landrigan 2006)

*** Positive hit in other ESNATS test battery test systems (Zimmer et al. 2014)

[a] Patejdl et al. 2013

[b] Orr and Ahlskog 2009

[c] Reboli and Mandler 1992

[d] Rinne et al., 2012

[e] Krug et al. 2013

[f] Grandjean and Herz 2015

[g] Eskenazi et al. 2013

[h] Grandjean and Landrigan 2006

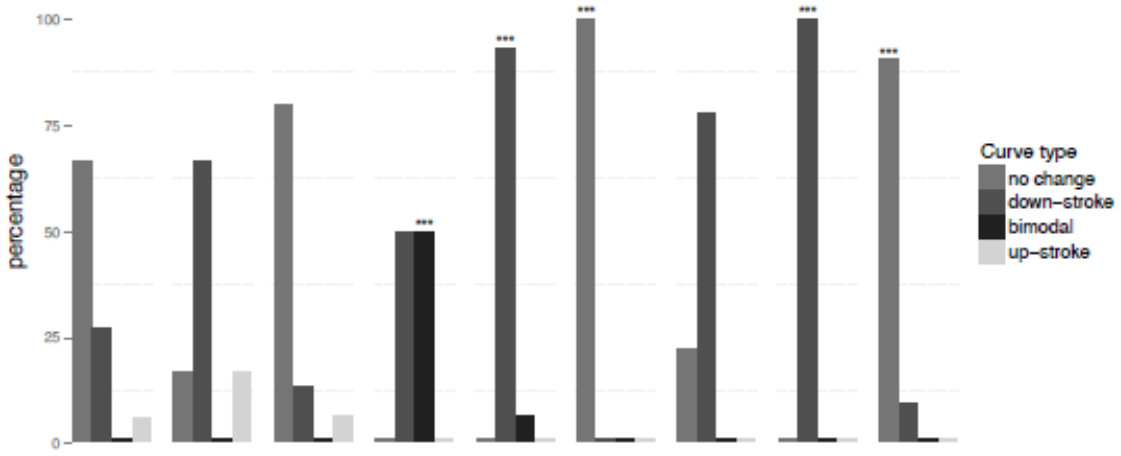
[i] Grandjean and Herz 2015

[l] Kreyberg et al. 1992

[m] Morris et al. 2009

[n] It is inhibiting signal cascades important in development, and it could therefor cause DNT. Most tyrosine kinase inhibitors are recommended not to be used during pregnancy. Some evidences of *in vivo* fetotoxicity for gefitinib were reported in a European public assessment report by the European Medicines Agency (EMA, 2008) and by Food and Drug Administration (FDA, 2015)

A


ESC
percentage
100
75
50
25
0
Curve type
no change
down−stroke
bimodal
up−stroke

B

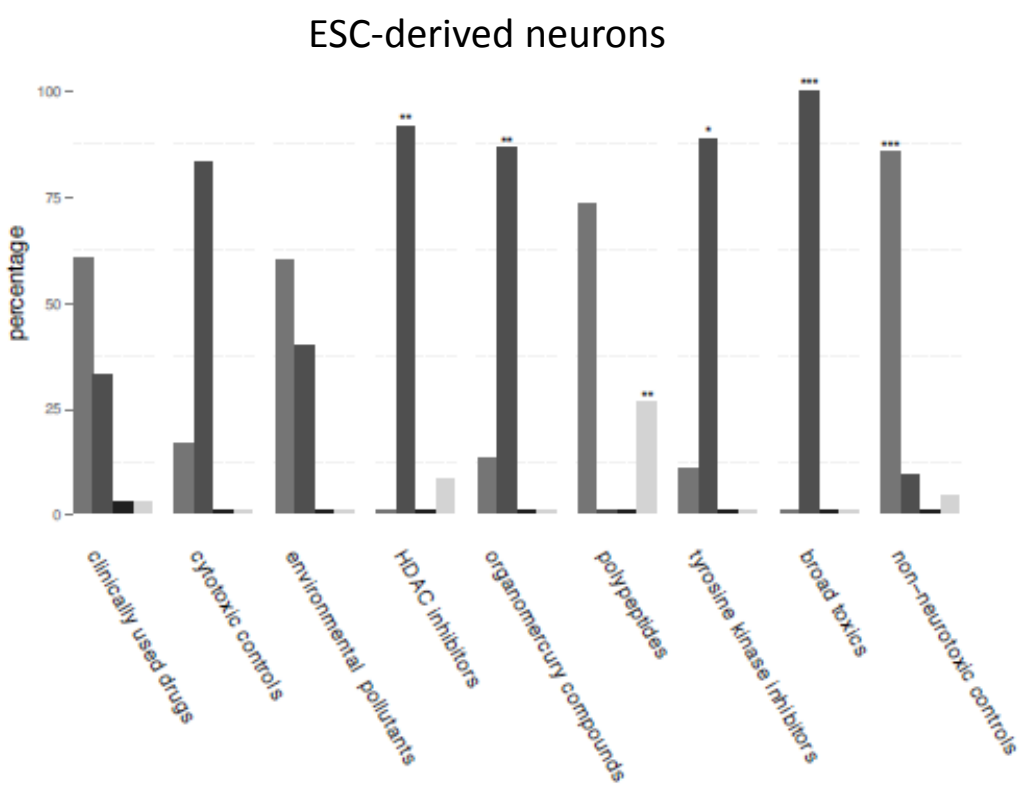

ESC-derived neurons
percentage
100
75
50
25
0
clinically used drugs
cytotoxic controls
environmental pollutants
HDAC inhibitors
organomercury compounds
polypeptides
tyrosine kinase inhibitors
broad toxics
non−neurotoxic controls

Fig. S1

A	Rat plasma	Human plasma	N2 medium (x)	BHK medium (y)
Lipid fraction (VFL) [mg/l]	3600	6000	-	160
Albumin concentration (P) [µM]	421	600	-	36
Total protein concentration (P)* [µM]	877	1000	10.9	63

B	Kow	fb,pl	MW
Abiraterone	131826	0.998	349.5 g/mol

Concentrations in [µM]	Rat		Human	
	C_{max}	C_{avg}	C_{max}	C_{avg}
Total concentration *in vivo (ECpl)*	3.09E-02	4.01E-03	6.47E-01	1.40E-01
Free concentration *in vivo*	1.30E-07	1.68E-08	1.63E-06	3.53E-07
Equivalent total concentration *in vitro (ECx)*	3.83E-04	4.97E-05	7.04E-03	1.52E-03
Equivalent total concentration *in vitro (ECy)*	2.22E-03	2.88E-04	4.07E-02	8.80E-03

C	Kow	fb,pl	MW
Geldanamycin	417	0.990	560.7 g/mol

Concentrations in [µM]	Rat
	C_{max}
Total concentration *in vivo (ECpl)*	2.23E-00
Free concentration *in vivo*	8.91E-03
Equivalent total concentration *in vitro (ECx)*	8.91E-03
Equivalent total concentration *in vitro (ECy)*	1.98E-01

D	Kow	fb,pl	MW
Teriflunomide	327	0.995	270.2 g/mol

Concentrations in [µM]	Rat		Human
	C_{max}	C_{avg}	C_{avg}
Total concentration *in vivo (ECpl)*	1.11E+01	5.09E+00	1.65E+02
Free concentration *in vivo*	2.56E-02	1.17E-02	2.80E-01
Equivalent total concentration *in vitro (ECx)*	2.56E-02	1.17E-02	2.80E-01
Equivalent total concentration *in vitro (ECy)*	9.72E-01	4.45E-01	1.01E-01

Fig. S2

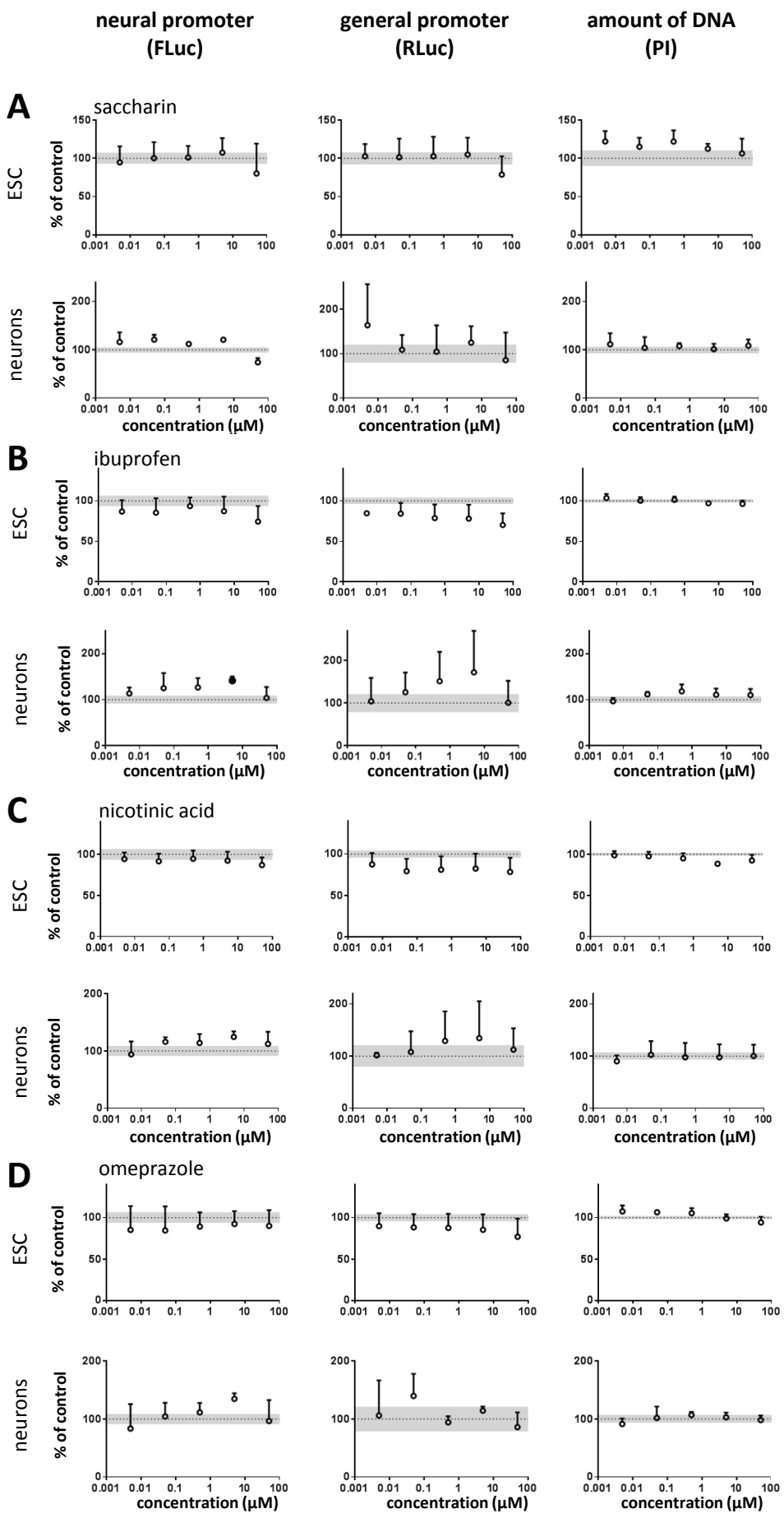

neural promoter (FLuc)
general promoter (RLuc)
amount of DNA (PI)
A
saccharin
B
ibuprofen
C
nicotinic acid
D
omeprazole
ESC
neurons
% of control
concentration (µM)

Fig. S3

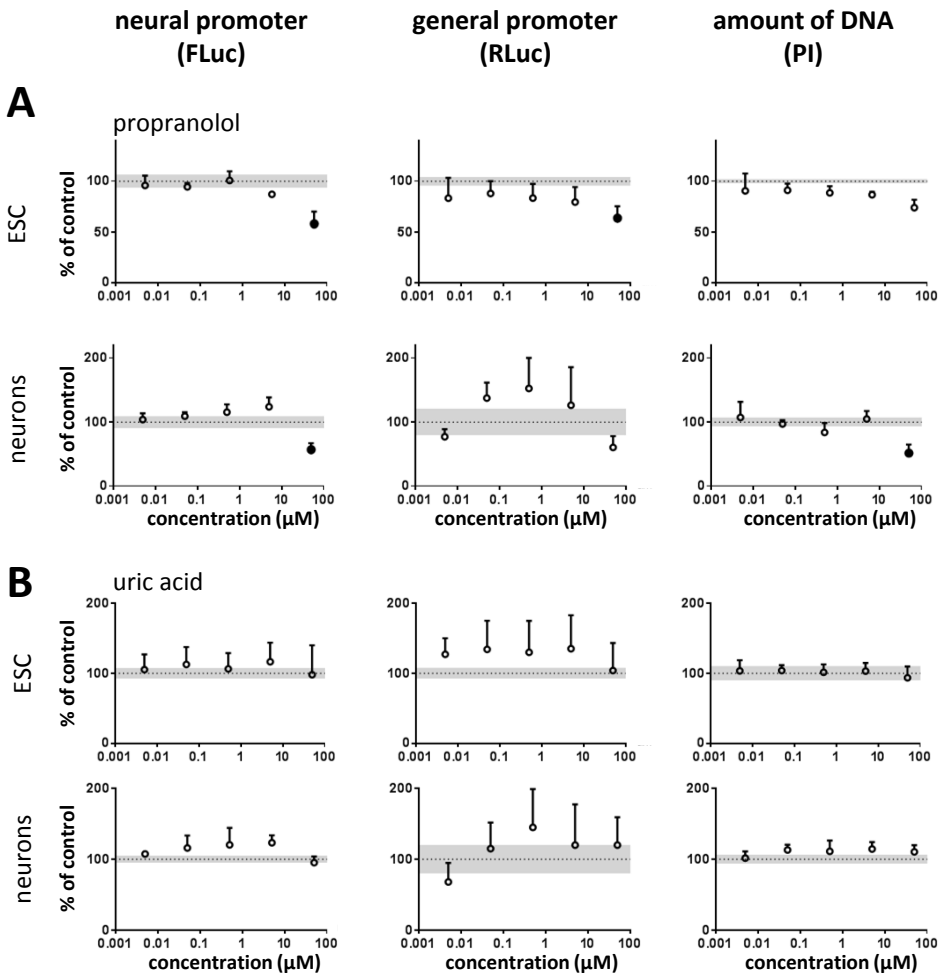

neural promoter (FLuc)
general promoter (RLuc)
amount of DNA (PI)
A
propranolol
ESC
neurons
% of control
concentration (µM)
B
uric acid
ESC
neurons
% of control
concentration (µM)

Fig. S4

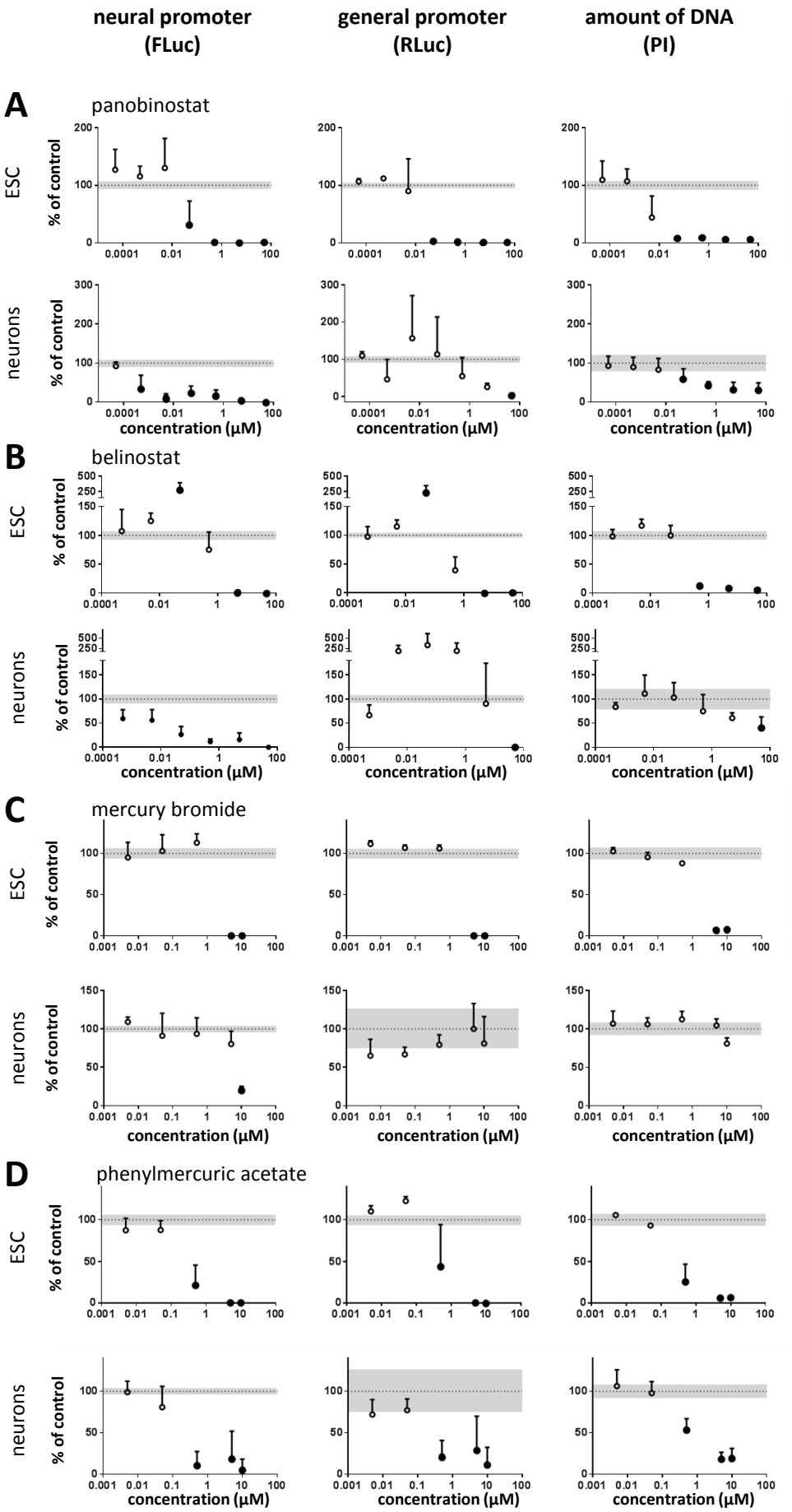

neural promoter (FLuc)
general promoter (RLuc)
amount of DNA (PI)
A
panobinostat
B
belinostat
C
mercury bromide
D
phenylmercuric acetate
ESC
neurons
% of control
concentration (μM)

Fig. S5

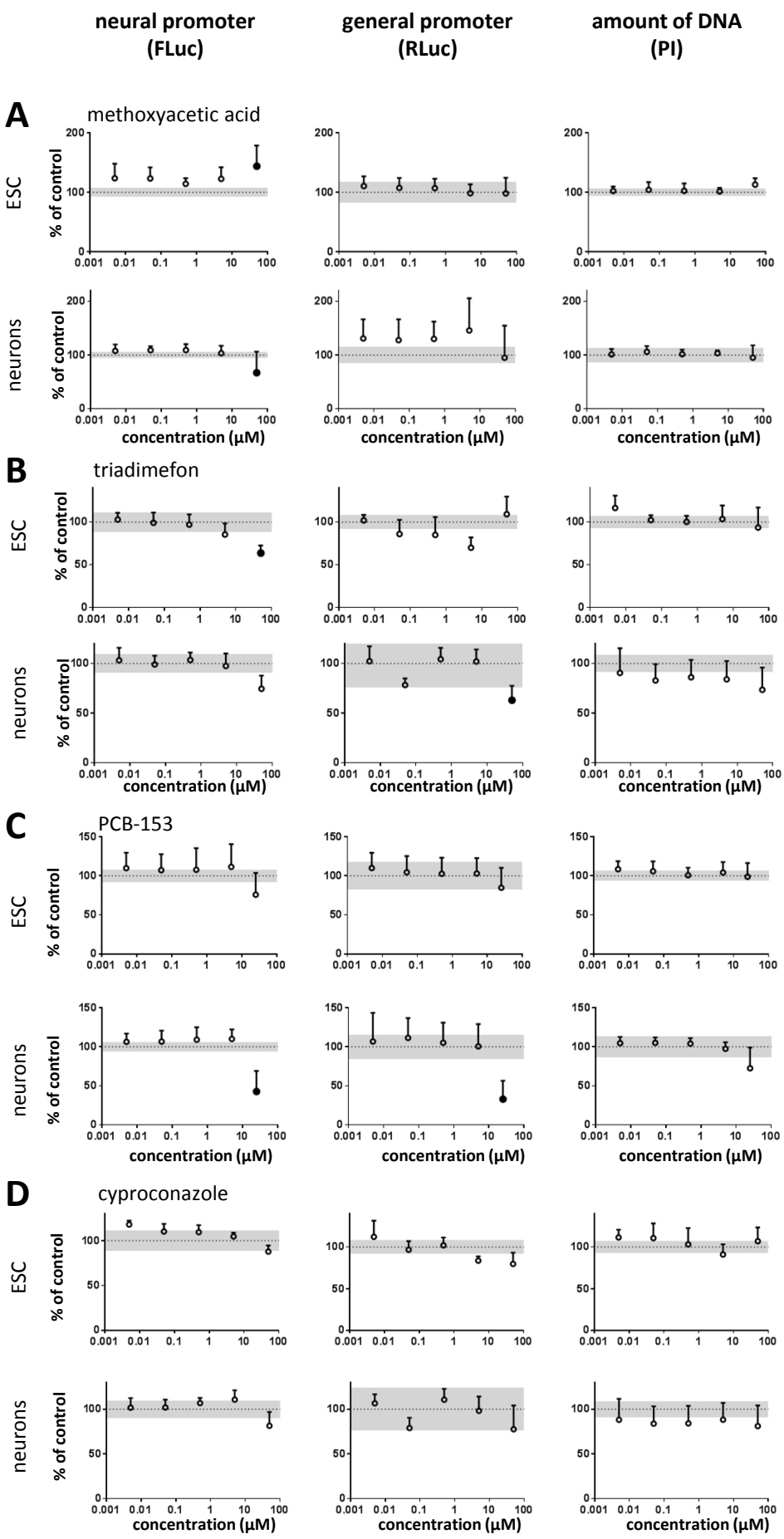

neural promoter (FLuc)
general promoter (RLuc)
amount of DNA (PI)
A
methoxyacetic acid
B
triadimefon
C
PCB-153
D
cyproconazole
ESC
neurons
% of control
concentration (µM)

Fig. S6

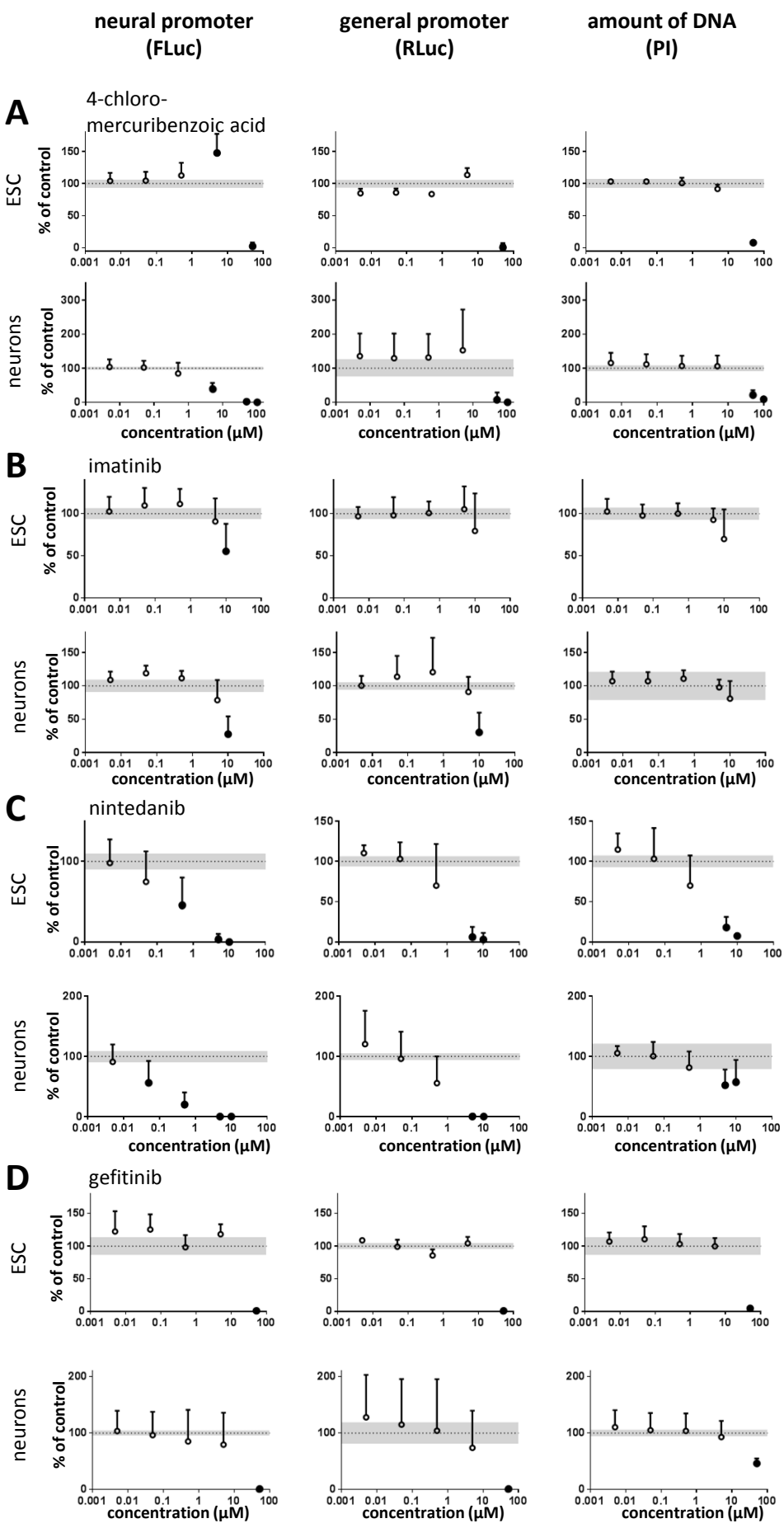

neural promoter (FLuc)
general promoter (RLuc)
amount of DNA (PI)
A
4-chloro-mercuribenzoic acid
B
imatinib
C
nintedanib
D
gefitinib
ESC
neurons
% of control
concentration (µM)

Fig. S7

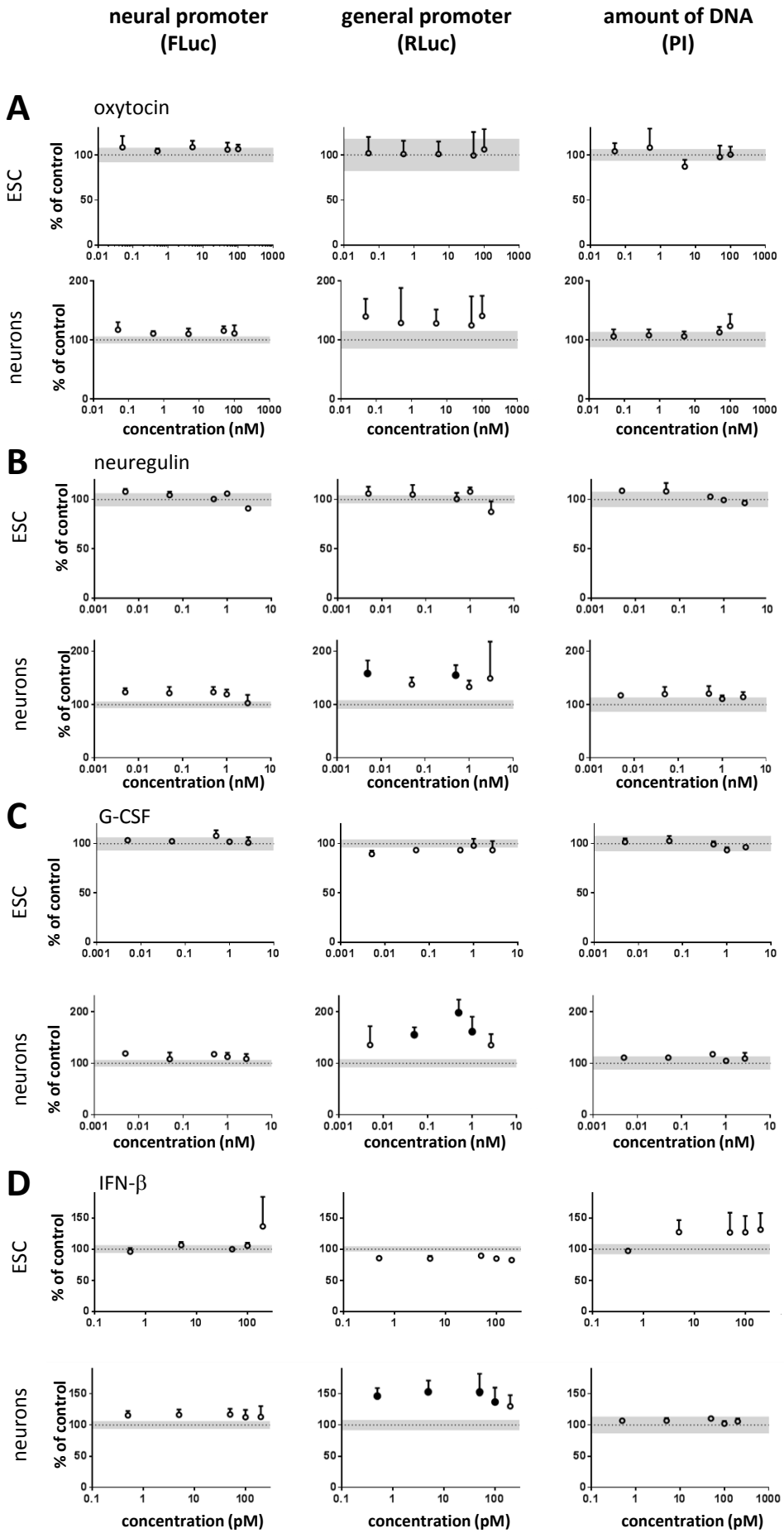

neural promoter (FLuc)
general promoter (RLuc)
amount of DNA (PI)
A
oxytocin
B
neuregulin
C
G-CSF
D
IFN-β
ESC
neurons
% of control
concentration (nM)
concentration (pM)

Fig. S8

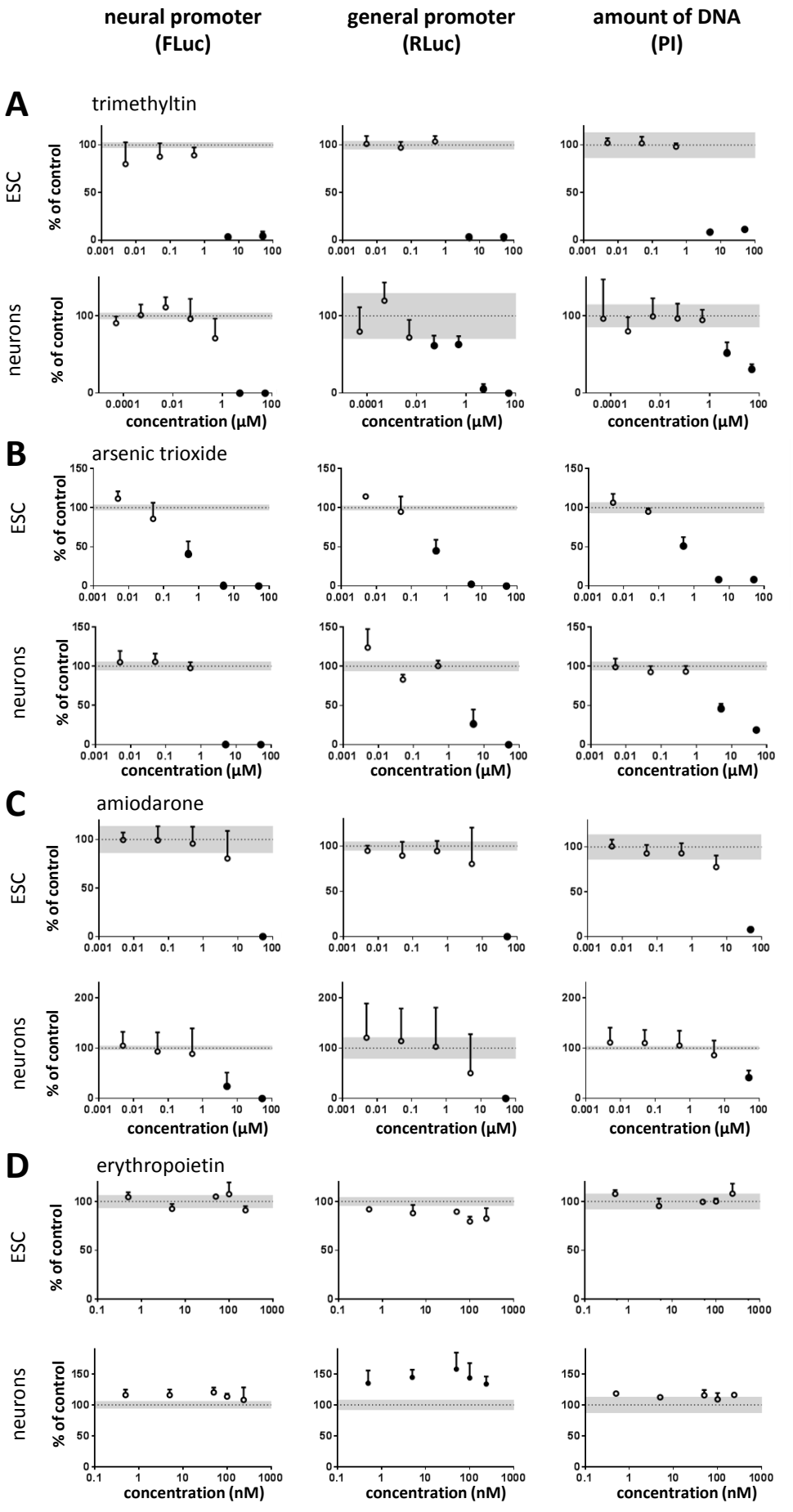

neural promoter (FLuc)
general promoter (RLuc)
amount of DNA (PI)
A
trimethyltin
B
arsenic trioxide
C
amiodarone
D
erythropoietin
ESC
neurons
% of control
concentration (µM)
concentration (nM)

Fig. S9

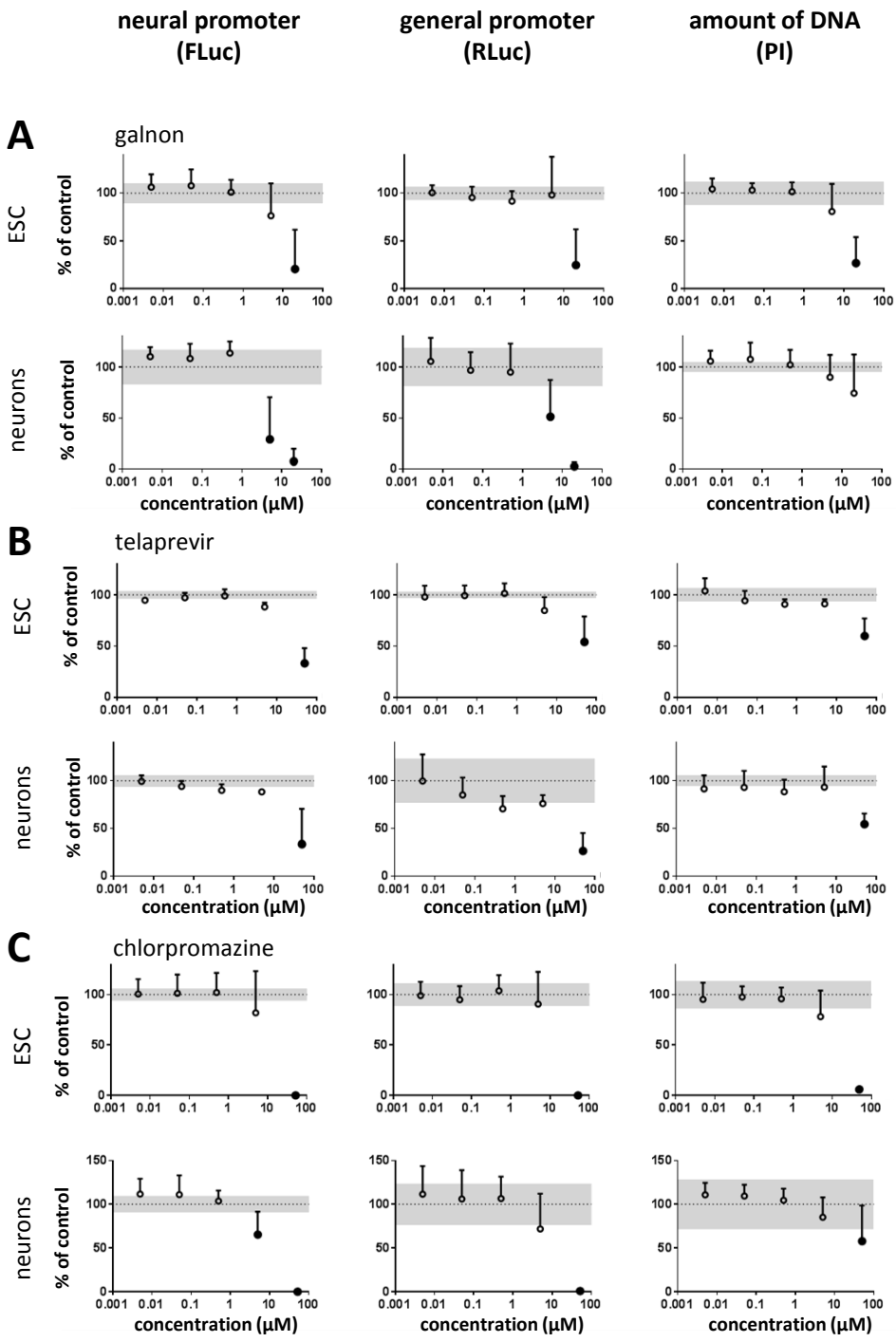

neural promoter (FLuc)
general promoter (RLuc)
amount of DNA (PI)
A
galnon
B
telaprevir
C
chlorpromazine
ESC
neurons
% of control
concentration (µM)

Fig. S10

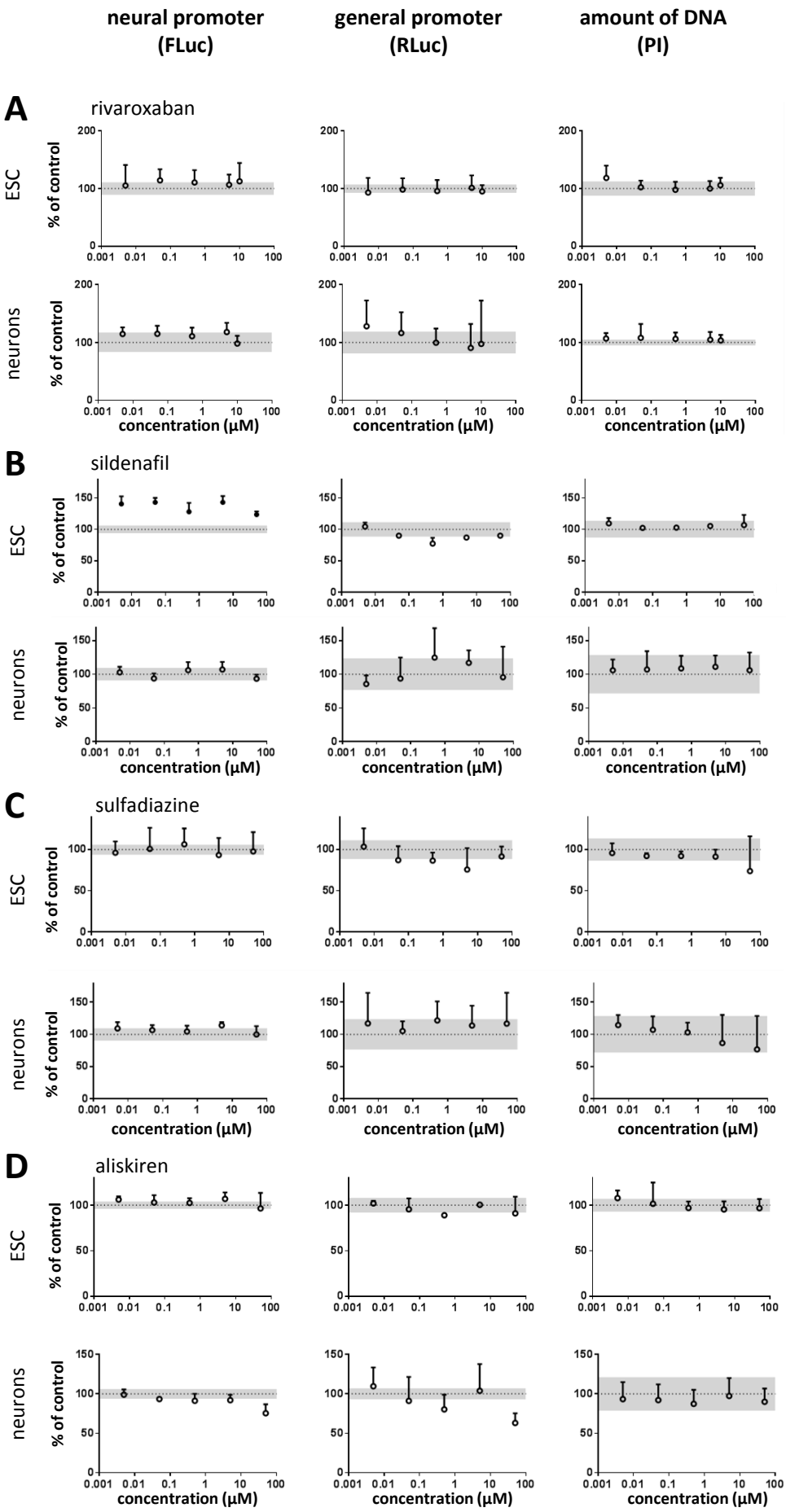

neural promoter (FLuc)
general promoter (RLuc)
amount of DNA (PI)
A
rivaroxaban
B
sildenafil
C
sulfadiazine
D
aliskiren
ESC
neurons
% of control
concentration (µM)

Fig. S11

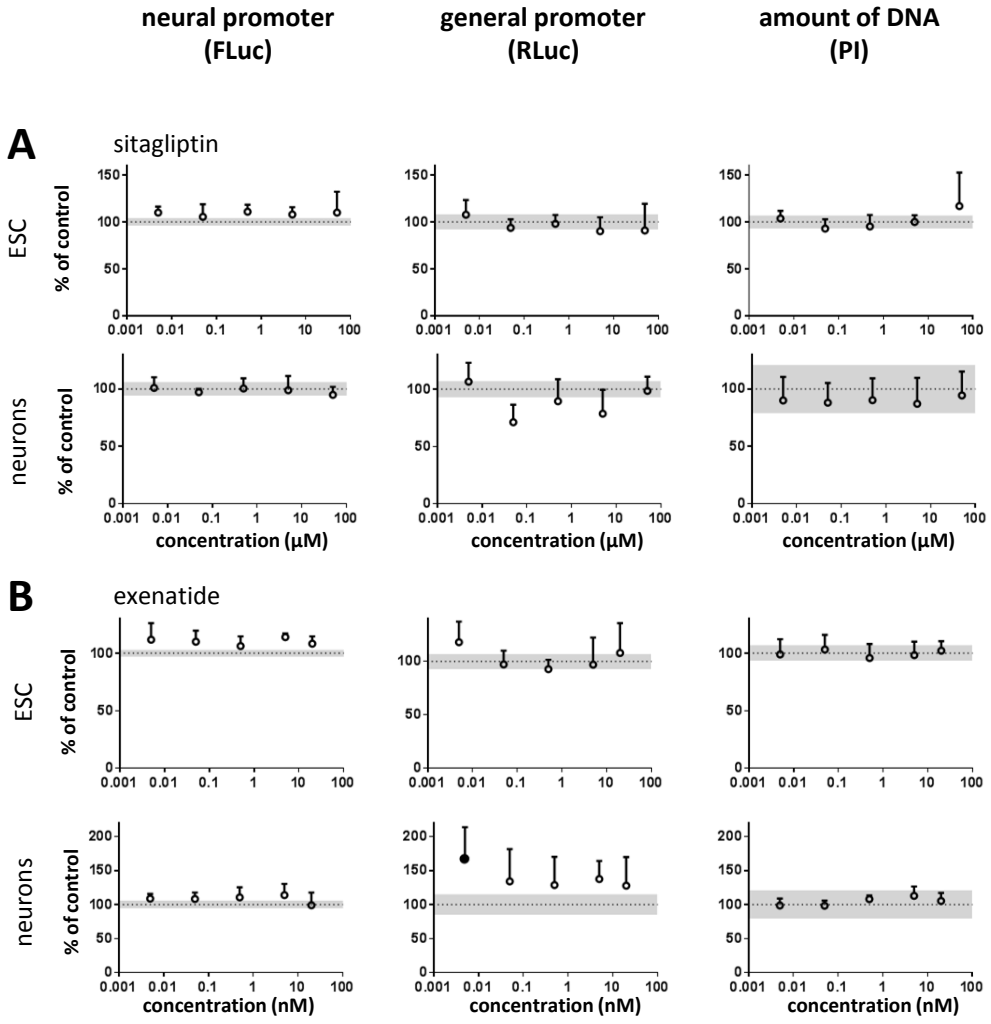

neural promoter (FLuc)
general promoter (RLuc)
amount of DNA (PI)
A
sitagliptin
ESC
neurons
% of control
concentration (µM)
B
exenatide
concentration (nM)

Fig. S12

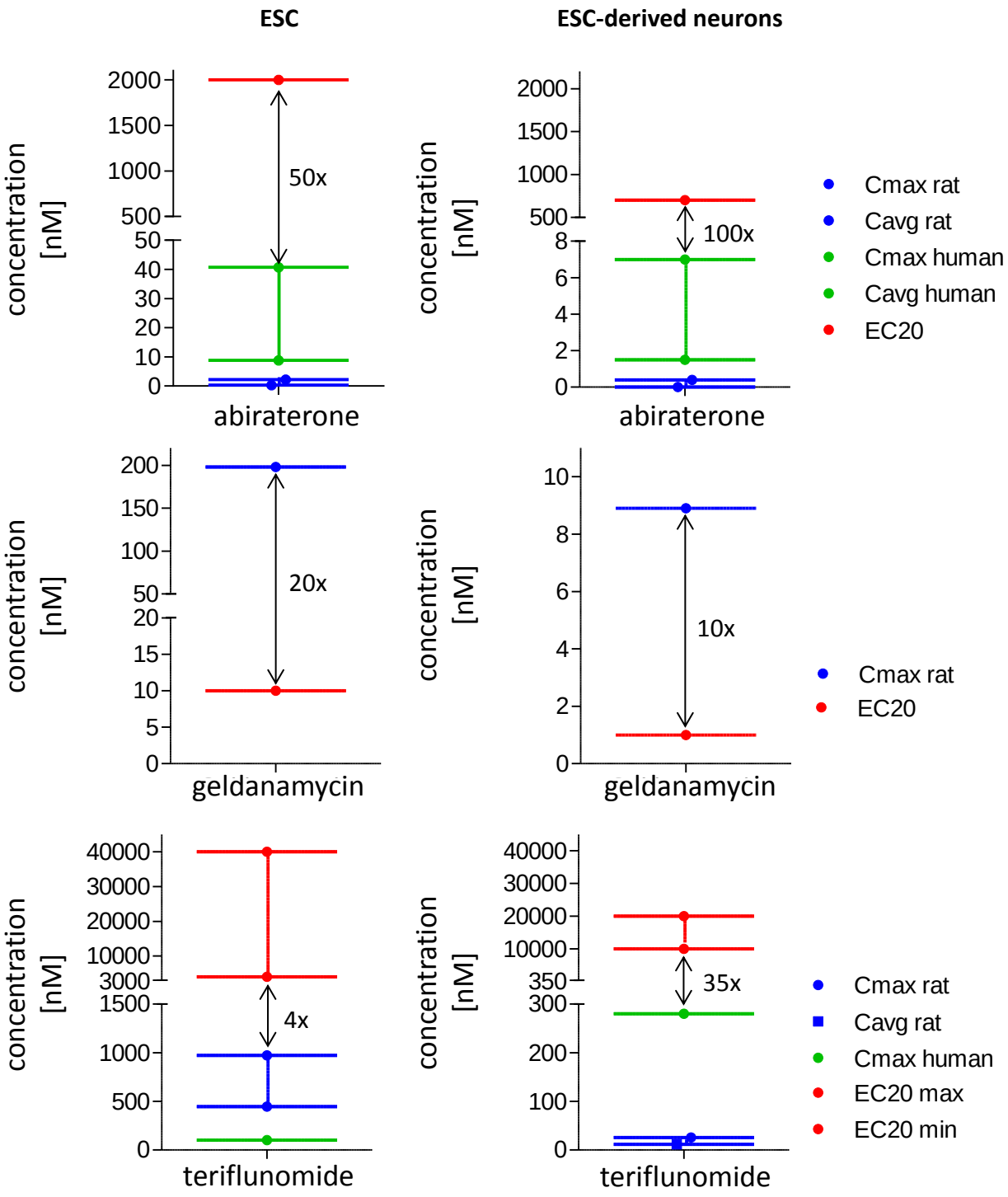

ESC
ESC-derived neurons
concentration [nM]
2000
1500
1000
500
50
40
30
20
10
0
50x
abiraterone
8
6
4
2
100x
200
150
100
20x
geldanamycin
10
5
10x
40000
30000
20000
10000
3000
1500
4x
teriflunomide
350
300
200
100
35x
Cmax rat
Cavg rat
Cmax human
Cavg human
EC20
EC20 max
EC20 min

Fig. S13

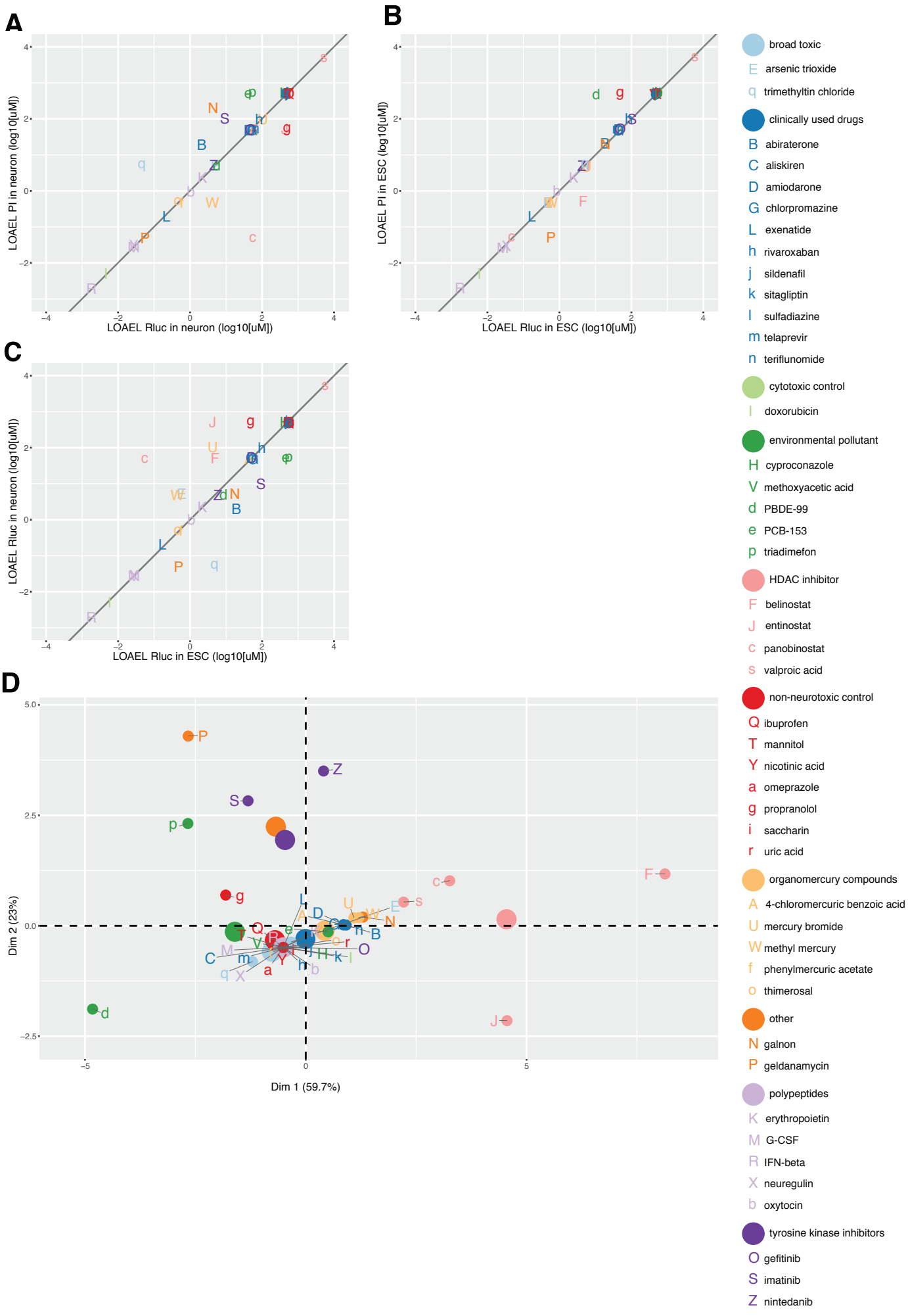

A
LOAEL PI in neuron (log10[uM])
LOAEL Rluc in neuron (log10[uM])
B
LOAEL PI in ESC (log10[uM])
LOAEL Rluc in ESC (log10[uM])
C
LOAEL Rluc in neuron (log10[uM])
LOAEL Rluc in ESC (log10[uM])
D
Dim 2 (23%)
Dim 1 (59.7%)
broad toxic
E arsenic trioxide
q trimethyltin chloride
clinically used drugs
B abiraterone
C aliskiren
D amiodarone
G chlorpromazine
L exenatide
h rivaroxaban
j sildenafil
k sitagliptin
l sulfadiazine
m telaprevir
n teriflunomide
cytotoxic control
I doxorubicin
environmental pollutant
H cyproconazole
V methoxyacetic acid
d PBDE-99
e PCB-153
p triadimefon
HDAC inhibitor
F belinostat
J entinostat
c panobinostat
s valproic acid
non-neurotoxic control
Q ibuprofen
T mannitol
Y nicotinic acid
a omeprazole
g propranolol
i saccharin
r uric acid
organomercury compounds
A 4-chloromercuric benzoic acid
U mercury bromide
W methyl mercury
f phenylmercuric acetate
o thimerosal
other
N galnon
P geldanamycin
polypeptides
K erythropoietin
M G-CSF
R IFN-beta
X neuregulin
b oxytocin
tyrosine kinase inhibitors
O gefitinib
S imatinib
Z nintedanib

Fig. S14

Supplemental Figure Legends

Fig. S1 Type of response curves as a function of compound category. Four types of response curves were observed: no change, down-stroke, bimodal, upstroke. Histograms representing the percentage of each curve type in a compound category are shown. Separate analysis for pluripotent cells (panel A) and neurons (panel B). Significant enrichment of a response type in a compound category was estimated with hypergeometric probability distribution (* FDR < 0.05; ** FDR < 0.01; *** FDR < 0.001). FDR = false discovery rate (i.e. p-value corrected with Benjamin & Hochberg approach).

Fig. S2 (A) Values for lipid fraction (VFL), albumin concentration (P) and total protein concentration (P)* in rat and human plasma and in N2 (x) and BHK (y) medium. The total protein concentration was used for abiraterone IVIV-modelling only. (B-D) Values for plasma bound fraction (fb,pl); octanol:water partition coefficient (Kow) and molecular weight (MW). For each compound, the total concentration found to be toxic (or effective) in *in vivo* studies and the related free concentration are reported for different species (rat and human). Two parameters have been used: maximal concentration (Cmax) and average concentration (Cavg) calculated as the ratio of the area under the curve (AUC) on the dose interval (τ). These data have been used to calculate equivalent total (nominal) *in vitro* concentrations corresponding to *in vivo* toxic/effective concentrations for N2 medium (ECx) and BHK medium (ECy).

Fig. S3 and S4 Effect of well-known non-toxic compounds. Cells were exposed to compounds for 48h, neural (FLuc) and general (RLuc) promoter activities and DNA quantity (PI assay) were determined. Results are expressed as percent of control + SD. Mean control values (100%) is shown as dotted line; the SD of control values is shown as grey area. Data points that differ in a statistically significant manner from control values were determined by one-way repeated-measures ANOVA followed by Dunnett's post hoc test and are shown as filled circles. Data were obtained from 4-6 replicates.

Fig. S5 - S12 Effect of other compounds of ESNATS collection. Cells were exposed to compounds for 48h, neural (FLuc) and general (RLuc) promoter activities and DNA quantity (PI assay) were determined. Results are expressed as percent of control + SD. Mean control values (100%) are shown as dotted line; the SD of control values is shown as grey area. Data points that differ in a statistically significant manner from control values were determined by one-way repeated-measures ANOVA followed by Dunnett's post hoc test and are shown as filled circles. Data were obtained from 4-14 replicates.

Fig. S13 Graphical representation of the relevant concentrations listed in Fig.1 B (main text). Data in rats (blue), in clinical studies (green) and in our *in vitro* systems (ESC and neurons) are showed for each compound. The arrows indicate the concentration folds between the *in vitro* and the *in vivo* concentration ranges.

Fig. S14 A-C) A scatterplot of log10 transformed LOAEL. D) A principal component analysis (PCA) of compounds using orthogonal distances from the alert plot diagonals. For each compound orthogonal distances to the diagonals on alert plots (A-G) in the figure 6 were calculated, yielding 6 values for each compound. These were used as input for principal component analysis (PCA) where each dot represents a compound that was labeled according to its class in Table 2.

Supplemental Bibliography

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