Small molecule screening identifies regulators of the transcription factor DeltaFosB

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Supplemental Materials

Supplemental Methods

Protein overexpression and purification. To produce ΔFosB or JunD homodimers, insect cells at ca. 1.5 x 10^6 cells ml^{-1} were infected at MOI 1.5. To produce the heterodimer ΔFosB/JunD, insect cells at ca.1.5 x 10^6 cells ml^{-1} were infected by adding roughly equal amounts of each virus (MOI 1.5 for each virus). To purify ΔFosB, 6L cell pellets were lysed in 25 mM Tris pH 8, 0.2 % Triton X-100, 1 mM TCEP with Complete EDTA-protease inhibitors (Roche), treated with DNAse, and the lysate clarified through centrifugation. After increasing the NaCl concentration to 1M and adding 0.5 M NaBr, the cleared supernatant was batch bound to 15 ml bed volume of Ni-chelating His-bind resin (Novagen) for 3 hours, washed with buffer A (25 mM Tris pH 8, 1 M NaCl, 20 mM imidazole), and eluted with a gradient of buffer B (25 mM Tris pH 8, 1 M NaCl, 500 mM imidazole). The Ni-eluate was dialyzed in two steps to lower the NaCl concentration (into buffer 25 mM Tris pH 9, 75 mM NaCl, 1 mM DTT, 0.5 mM PMSF), applied to a MonoQ column (GE Healthcare) and eluted with a gradient (buffer 25mM Tris pH 9, 1M NaCl, 1mM DTT). As a final step, the protein was subject to size exclusion chromatography using a HiLoad Superdex 200 16/60 gel filtration column equilibrated with 20 mM Tris pH 7.5, 1 M NaCl. The protein was stored in 20 mM Tris pH 7.5, 1 M NaCl, aliquotted and flash-frozen. ΔFos/JunD and JunD were purified in the same way, except that prior to Ni-NTA, the NaCl concentration of the cell lysates were increased to only 0.5 NaCl and NaBr was omitted, the buffers for Ni chromatography contained only 0.5 M NaCl, and the final size exclusion column was run in 20 mM Tris pH 8, 1 M NaCl. ΔFosB homodimer was also purified using this milder procedure as a control and showed no detectable difference in DNA binding properties compared to the purification procedure involving high NaCl concentrations and NaBr. Protein purity was assessed with SDS-PAGE gels. Protein concentrations were determined using the Biorad Assay.

Cell toxicity assays. Mouse Neuro2A neuroblastoma cells (ATCC) were maintained in Eagle's Minimum Essential Medium (EMEM) (ATCC), supplemented with 10% fetal

bovine serum (ATCC), at 37˚C and 5% CO_2 on 10 cm plates (Corning, #430167). Neuro2A cells were plated onto 96-well plate dishes (Corning, cat. No3599, at 1 x 10^6 cells/dish) in 100 μl-EMEM without anti-biotics. The next day, compounds (50 mM in DMSO) were added to the cells in a concentration series (3.125 μM, 6.25 μM, 12.5 μM, 25 μM and 50 μM) resulting in a final DMSO concentration of 0.1%, in quadruplicate. Cells were incubated with compound for 48 hr. As a control, cells were also incubated in presence of 0.1% DMSO, but with no compound. At the end of each incubation, 100 μl CellTiter-Glo Reagent was added to the culture medium present in each well and the luminescence activity was measured using an LMax II384 microplate reader.

Transactivation assay. For the luciferase assay, cells were harvested two days after transfection with 4×AP-1/RSV-Luc and ΔFosB-pcDNA3.1 (or alternatively FosB, JunD, ΔFosB/JunD or FosB/JunD), by trypsin and collected in EMEM. Cells were subsequently plated onto 24-well plates (Corning, #3524) at cell density 8.5 x 10^4 cells/wells (i.e., 60% confluency) and the next day, compounds were added directly to the medium. After incubation with compounds for 48 hr, the cells were washed in PBS and lysed by adding 100 μl Passive Lysis buffer (1X, Promega) to the cell monolayer in each well. Cell lysates were transferred to a 96-well white plate (Corning, #3912). Luciferase activity was measured directly from the 96-well white plate by detecting luminescence using an LMax II384 microplate reader. As a control for the effect of DMSO, Neuro2A cells were exposed to 0.1% DMSO (i.e., no compound). The cellular protein concentration of each well was assessed by measuring the protein concentration of 10 μl cell lysate using 660 nm protein assay reagent (Pierce). The effect of compounds on the transcription factor-mediated luciferase activity was expressed as luciferase activity/μg protein in order to take into account any effects of the compounds on cell-growth, and is reported relative to wells containing cells exposed to DMSO alone. The maximum effect of the compounds was observable after 48 hr.

***In vivo* studies.** Alzet micro-osmotic pumps were installed bilaterally with cannulae into the nucleus accumbens of individually housed mice (200 μL per hemisphere). Solutions

of 100 μM compound C2 (7 mice), 100 μM of a structurally related but inactive analogue (Chembridge 5996481, 8 mice) or the vehicle (0.5% DMSO in PBS, 6 mice) were administered via the pumps for 14 days. During the last 7 days of compound administration, all mice received additionally a daily intraperitoneal injection of cocaine at 10 mg/kg weight. Mice were sacrificed 24 hours after the last dose of cocaine.

The effect of compound administration on mRNA levels of two known target genes for ΔFosB, GluR2 and cdk5,[1] was analyzed by qPCR. Mice were killed by decapitation, and 15-gauge NAc punches were taken bilaterally, homogenized in Trizol, and processed according to the manufacturer's instructions. RNeasy Micro columns (QIAGEN) were used to purify RNA. RNA A260/280 and A260/230 ratios were confirmed by spectroscopy measurements to be >1.8. iScript cDNA synthesis (Bio-Rad) was used to reverse transcribe RNA into cDNA and ~2.5 ng of cDNA was used for each quantitative PCR (qPCR) reaction, in addition to primers and SYBR Green. Each reaction was run in triplicate and the qPCR data analyzed with the ΔΔCt method using expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a normalizing control, as previously described.[2] Primers used in qPCR reactions were: GAPDH (5'-AGGTCGGTGTGAACGGATTTG-3' and 5'-TGTAGACCATGTAGTTGAGGTCA-3'), GluR2 (5'-GCCGAGGCGAAACGAATGA-3' and 5'-CACTCTCGATGCCATATACGTTG-3'), and cdk5 (5'-GTCCATCGACATGTGGTCAG-3' and 5'-CTGGTCATCCACATCATTGC-3').

Circular Dichroism (CD). ΔFosB protein (0.5 mg/ml) was dialyzed against CD buffer (12.5 mM phosphate buffer, pH 8, 50 mM NaF and 1 mM DTT) overnight at 4^{o} C using a Slide-a-Lyzer (Pierce, MWCO 8000 Da). A Bio-Rad protein assay was used to determine the protein concentration after dialysis. Reagents were prepared and combined in CD buffer, i.e., the protein sample (ΔFosB at 0.1 mg/ml), compounds (100 μM C2 or C6 prepared from a 2.5 mM stock in ethanol) or ethanol (as a control for no compound), and cdk5 oligo (12.5 μM cdk5 made from a 500 μM stock in annealing buffer), and incubated at RT for 15 min. For each protein sample, a corresponding background sample was prepared that contained the identical sample components

including cdk5 oligo and compounds as necessary, but no protein. The compounds alone did not alter the CD spectra compared to the buffer, but DNA did. CD spectra were recorded at RT on an Aviv-202 spectrometer from 190 to 260 nm in a 1.0 mm path-length quartz cuvette using an average time of 2 seconds at the spectral bandwidth of 1.0 nm. An average spectrum was obtained from three individual scans and a final background cleared spectrum was obtained by subtracting the background spectrum from the corresponding protein spectrum. An online protein analysis server 'dichroweb' was used to deconvolute the spectra as described in the main text. It is not known if DNA bound to protein and unbound DNA alter the spectra (and the background correction) similarly.

Supplemental References

(1) Nestler, E. J. (2008) Transcriptional mechanisms of addiction: role of DeltaFosB, *Philos. T. Roy. Soc. B: Biol. Sci. 363*, 3245–3255.

(2) Zachariou, V., Bolanos, C. A., Selley, D. E., Theobald, D., Cassidy, M. P., Kelz, M. B., Shaw-Lutchman, T., Berton, O., Sim-Selley, L. J., Dileone, R. J., Kumar, A., and Nestler, E. J. (2006) An essential role for DeltaFosB in the nucleus accumbens in morphine action, *Nat. Neurosci. 9*, 205–211.

Supplemental Legends

Figure S1. Toxicity of small molecule ΔFosB modulators. Compounds were tested in Neuro2A cells using a cell viability assay at 50 μM for C1, C2, C3, C4, C5 and C7, and at 12.5 μM for C6. Cell viability in presence of compound was expressed as a function of viability in presence of 0.1 % DMSO monitored after 48 hours.

Figure S2. Small molecule ΔFosB modulators alter the transcription of a reporter gene in cell-based transactivation assays. Neuro2A cells were co-transfected with a luciferase reporter gene (4xAP-1/RSV-Luc) and one of the following: FosB, FosB and JunD, or JunD. Transactivation of the reporter gene was monitored after 48 hours of incubation with 50 μM C1, C2, or C7, 25 μM C4, 12.5 μM C6 or as a control 0.1 % DMSO. The change in luciferase signal is expressed as luciferase units per total cellular protein (LU/μg).

Figure S3. Changes in the secondary structure of ΔFosB with increasing amount of compound C2 and C6. a) CD spectra of ΔFosB alone, and ΔFosB with 5 μM C2, 15 μM C2, and 100 μM C2. b) CD spectra of ΔFosB alone, and ΔFosB with 10 μM C6, 30 μM C6, and 100 μM C6.

Figure S4. Specificity of small molecule ΔFosB modulators for JunD homodimers. a) JunD homodimer binding to TMR-cdk5 under low salt conditions (50 mM NaCl, ●) and high salt conditions (175 mM NaCl, ▲). Binding is also shown for TMR-SCR (50 mM NaCl, ○) and (175 mM NaCl, △). Under high salt conditions, JunD binds TMR-cdk5 specifically though not as efficiently, and the binding of JunD to TMR-SCR (△) is suppressed.; b) Dose response curves of C1, C2 and C6 with JunD under low salt conditions (50 mM NaCl, left panel) and high salt conditions (175 mM NaCl, right panel). C1, C2 and C6 were tested in dose response assays as described in Fig. 3 without

protein (▽), in presence of 280 nM ΔFosB (◇) and in presence of 280 nM JunD (■). The positive control (oligo alone, ○) and the negative controls (ΔFosB, ∗) and (JunD+cdk5, □) are shown as well.

Table S1: Flow chart of high-throughput screening

Screen	Criteria to pass	Result
Primary Screen (1 dose at 15 μM)	• 3 SD decrease in FP signal compared to negative control	563 hits from 54,498 compounds
Confirmation Screen (8 point dose response curve)	• pIC_{50} > 5 (<10 μM) • commercially available	143 hits from 563 compounds 114 of 143 compounds available
Re-confirmation Screen fresh compounds (8 point dose response curve)	• decrease in FP signal with increasing compound concentration • no change in signal in absence of protein	78 hits from 114 compounds 11 hits from 78 compounds
Electrophoretic Mobility Assay (EMSA)	• disruption of ΔFosB:DNA complex with increasing compound concentration	8 hits from 11 compounds
Ethidium Bromide Displacement Assay (EBDA)	• < 25% decrease in fluorescent intensity up to 100 μM compound	5 out of 8 compounds
Toxicity Assay	• < 20% loss in cell viability at concentration used in transactivation assay • compound soluble in media	5 out of 5 compounds 4 out of 5 compounds
Transactivation Assay	• > 30% change in luciferase activity in presence of compound	3 out of 4 compounds

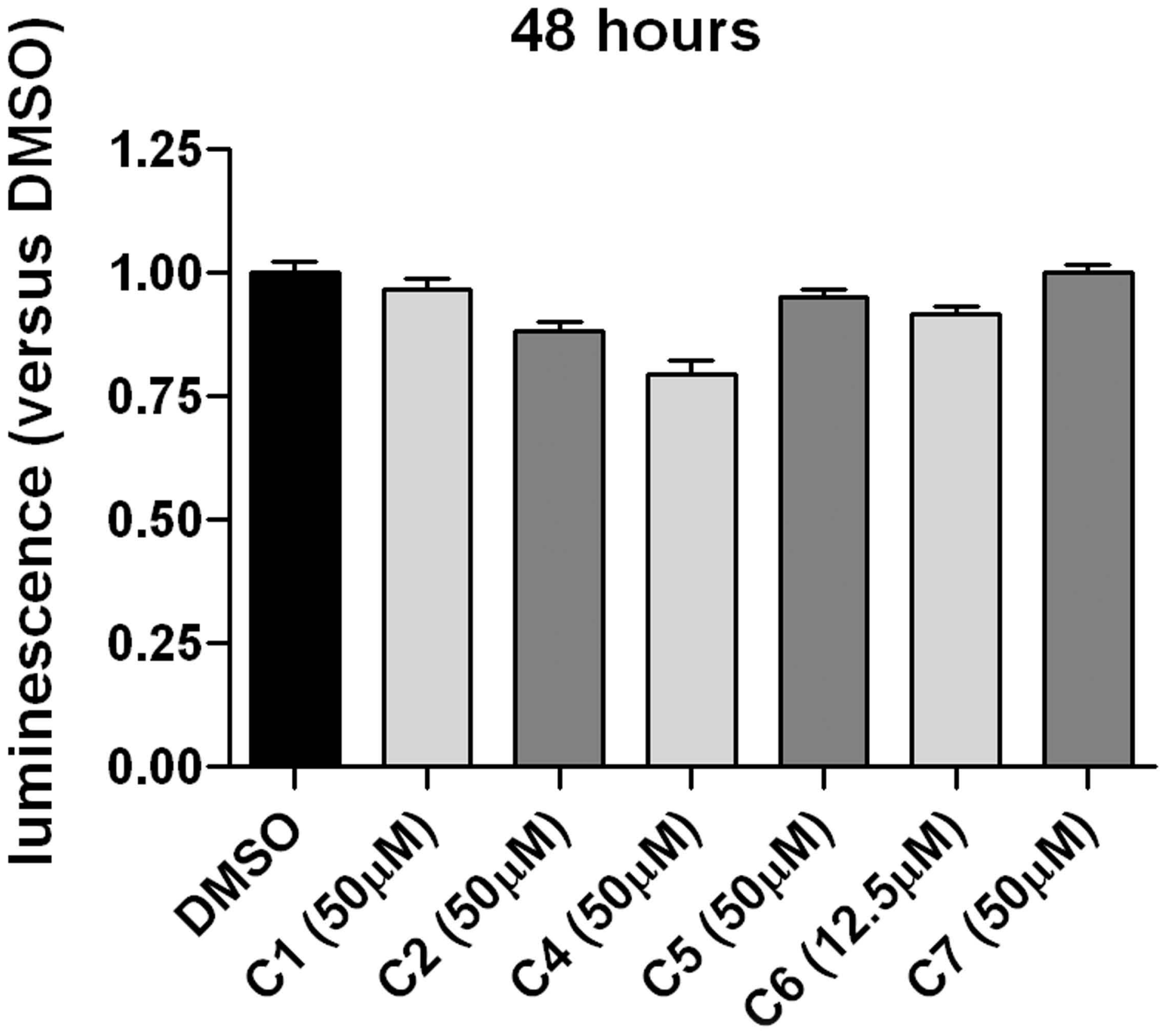
48 hours
luminescence (versus DMSO)
1.25
1.00
0.75
0.50
0.25
0.00
DMSO
C1 (50μM)
C2 (50μM)
C4 (50μM)
C5 (50μM)
C6 (12.5μM)
C7 (50μM)

Fig. S1

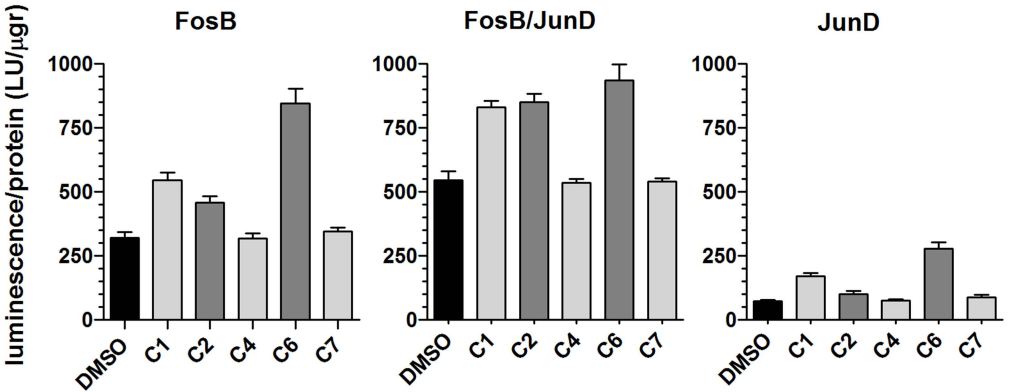
FosB
FosB/JunD
JunD
luminescence/protein (LU/μgr)
1000
750
500
250
0
DMSO
C1
C2
C4
C6
C7

Fig. S2

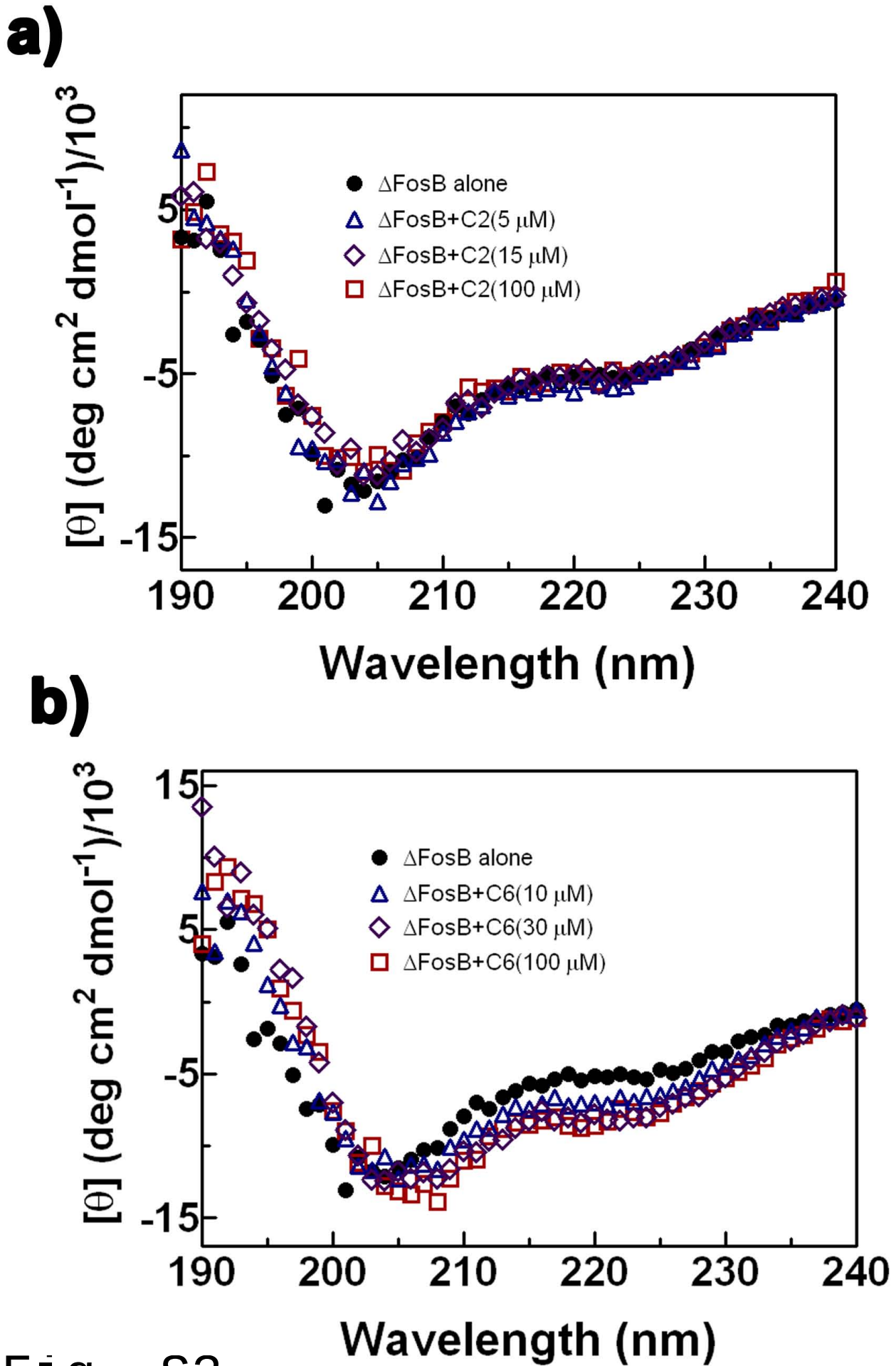
a)
[θ] (deg cm2 dmol-1)/10^3
5
-5
-15
190
200
210
220
230
240
Wavelength (nm)
ΔFosB alone
ΔFosB+C2(5 μM)
ΔFosB+C2(15 μM)
ΔFosB+C2(100 μM)
b)
[θ] (deg cm2 dmol-1)/10^3
15
5
-5
-15
190
200
210
220
230
240
Wavelength (nm)
ΔFosB alone
ΔFosB+C6(10 μM)
ΔFosB+C6(30 μM)
ΔFosB+C6(100 μM)

Fig. S3

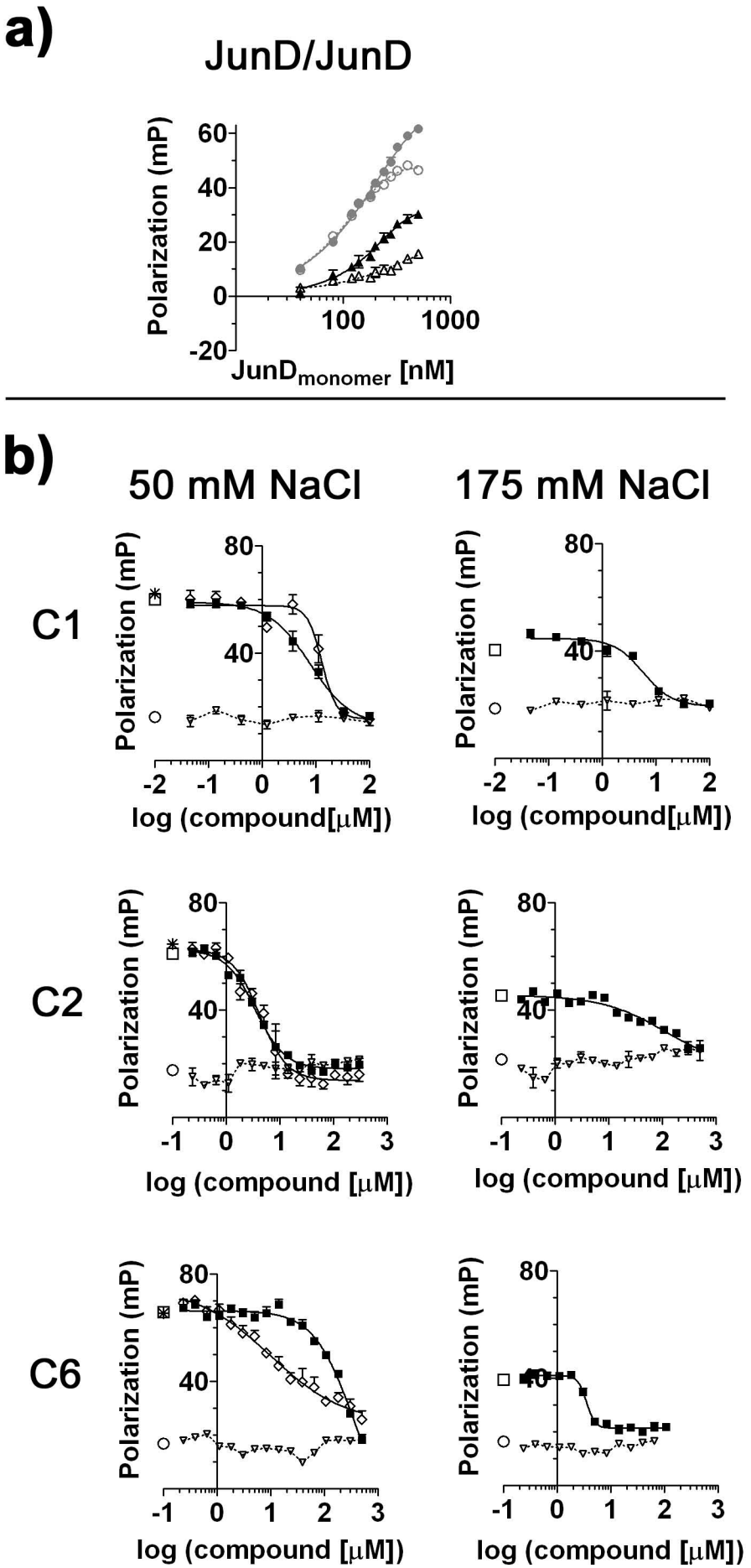
a)
JunD/JunD
Polarization (mP)
60
40
20
0
-20
100
1000
JunD monomer [nM]
b)
50 mM NaCl
175 mM NaCl
C1
C2
C6
Polarization (mP)
80
40
-2
-1
0
1
2
3
log (compound[μM])
log (compound [μM])

Fig. S4