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Identification of early gene expression changes in primary cultured neurons treated with topoisomerase I poisons

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Abstract

Topoisomerase 1 (TOP1) poisons like camptothecin (CPT) are currently used in cancer chemotherapy but these compounds can have damaging, off-target effects on neurons leading to cognitive, sensory and motor deficits. To understand the molecular basis for the enhanced sensitivity of neurons to CPT, we examined the effects of compounds that inhibit TOP1—CPT, actinomycin D (ActD) and β-lapachone (β-Lap)—on primary cultured rat motor (MN) and cortical (CN) neurons as well as fibroblasts. Neuronal cells expressed higher levels of *Top1* mRNA than fibroblasts but transcript levels are reduced in all cell types after treatment with CPT. Microarray analysis was performed to identify differentially regulated transcripts in MNs in response to a brief exposure to CPT. Pathway analysis of the differentially expressed transcripts revealed activation of ERK and JNK signaling cascades in CPT-treated MNs. Immediate-early genes like *Fos*, *Egr-1* and *Gadd45b* were upregulated in CPT-treated MNs. *Fos* mRNA levels were

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elevated in all cell types treated with CPT; *Egr-1*, *Gadd45b* and *Dyrk3* transcript levels, however, increased in CPT-treated MNs and CNs but decreased in CPT-treated fibroblasts. These transcripts may represent new targets for the development of therapeutic agents that mitigate the off-target effects of chemotherapy on the nervous system.

Keywords

motor neuron; cortical neuron; camptothecin; actinomycin D; β-lapachone; DNA damage response; immediate early gene; microarray; Dyrk3

INTRODUCTION

Topoisomerase I (TOP1) relaxes DNA supercoiling so that replication and gene transcription can proceed. TOP1 introduces single-strand DNA breaks, or nicks, in the supercoiled DNA by covalently binding to the DNA and forming a TOP1 cleavage complex (TOP1cc) [1]. Once the DNA is sufficiently unwound, the TOP1cc facilitates the religation of the nick and TOP1 is released from the DNA. TOP1ccs are normally very transient structures; camptothecin (CPT) and its derivatives act as interfacial inhibitors of TOP1 by reversibly stabilizing the complex. This stabilization prevents the religation of the double-stranded DNA [1] resulting in double-strand DNA breaks. As such, CPT-based compounds are sometimes referred to as TOP1 poisons as opposed to inhibitors [2]. Actinomycin D (ActD), in addition to its action as a transcription inhibitor [3], also stabilizes the TOP1cc and, therefore, acts as a TOP1 poison [4]. Alternatively, β-lapachone (β-Lap) is a direct inhibitor of TOP1 and does not interact with the TOP1cc [5].

Anti-cancer drugs like CPT and its derivatives can cause DNA damage and oxidative stress in non-target tissues like neurons [1;2]. The non-specific action of chemotherapy drugs on the central nervous system may contribute to chemotherapy-induced cognitive impairment, a phenomenon formerly known as “chemobrain” [6]. Dysarthria, which is an impairment of the motor component of speech, has been observed in cancer patients treated with the CPT prodrug irinotecan [7;8].

Neurons are exceptionally susceptible to DNA damaging agents like CPT because they maintain high levels of RNA synthesis and are more sensitive to oxidative stress [9]. We hypothesize that neurons differentially regulate the expression of genes that confer this increased susceptibility. In this study, we identified, by microarray, early transcripts differentially regulated in primary cultured rat neuronal (motor (MN) and cortical (CN) neurons) and non-neuronal (fibroblasts) cells after a brief exposure to the TOP1 inhibiting agents CPT, ActD and β-Lap.

MATERIALS AND METHODS

Ethics Statement

All animal experiments were conducted in accordance with the protocols described in the National Institutes of Health *Guide for the Care and Use of Animals* and were approved by

the Nemours Biomedical Research Institutional Laboratory Animal Care and Use Committee.

Embryonic Rat Primary Culture

Primary cultures of rat MNs, CNs and fibroblasts were obtained from embryonic day 15 (e15) Sprague-Dawley rat pups. Timed-pregnant dams (Charles River; Wilmington, MA) were deeply anesthetized using CO_2 and individual embryos were placed on ice in Hank's balanced salt solution (HBSS; Life Technologies, Grand Island, NY) after the uteri were remove. CNs and MNs were plated at a density of 5.0×10^4 cells/cm^2 on coverslips coated with 100 μg/mL poly-DL-ornithine (Sigma) and 2 μg/mL laminin (Millipore, Billerica, MA) for immunofluorescence or at a density of 2.5×10^6 cells/cm^2 on 10-cm dishes coated with poly-DL-ornithine and laminin for RNA extraction. Fibroblasts were plated at the same density but on gelatin-coated coverslips or 10-cm dishes.

For primary CNs and MNs, superficial segments of the forebrain or thoracolumbar spinal cords were dissected from embryos, rinsed in HBSS and incubated in 0.05% trypsin/EDTA (Life Technologies, Grand Island, NY) for 15 minutes at 37°C. After trypsinization, the cells were triturated in Neuron Medium (Neurobasal medium supplemented with 1% B27, 2% horse serum, 500 μM L-glutamine, 1% penicillin/streptomycin (pen/strept), (all from Life Technologies, Grand Island, NY), 25 mM β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO) and 1 ng/mL brain-derived neurotrophic factor (BDNF; R&D Systems, Minneapolis, MN)). MNs were separated from other spinal cord dissociated cells using Opti-Prep (Sigma-Aldrich; final concentration = 3%; 500xg for 15 minutes) and then centrifuged (300xg for 10 minutes) through a 4% bovine serum albumin (BSA; Sigma, St. Louis, MO) cushion. Both MNs and CNs were maintained in Neuron Medium.

Primary fibroblasts were harvested from the white tissues of the embryo by trypsinization. The pellets were resuspended in high glucose DMEM supplemented with 10% FBS, 2mM L-glutamine and 1% pen/strept. They were maintained in T-150 flasks for 2 passages prior to experiments.

These culturing conditions yielded high purities of the targeted cell types from embryonic rat tissues as shown using cell type-specific markers (β3-tubulin, neurofilaments and Fox3 for neurons, HB9 for MNs and fibronectin for fibroblasts; data not shown). In the primary CNs and MN cultures, there were very few (less than 5%) GFAP$^+$ glial cells present.

Drug Response Assays

Rat embryonic primary MNs, CNs and fibroblasts were treated with either DMSO (drug vehicle) or different concentrations of the following TOP1 inhibitors (n=3/dose/drug): camptothecin (CPT; Tocris Biosciences, Bristol, UK; 10 nM – 100 μM), β-lapachone (β-Lap, Sigma-Aldrich; 10 nM – 10 μM), and actinomycin D (ActD, Sigma-Aldrich; 10 nM – 10 μM). Primary cultures were treated for 24 hours starting at either 2 days in vitro (DIV2) or 5 days in vitro (DIV5).

Immunofluorescence

Cells were fixed with 4% paraformaldehyde in PBS, pH 7.4 for 15 minutes at room temperature. The cultures were then rinsed in PBS, permeabilized with 0.1% Triton-X100 (Sigma, St. Louis, MO) in PBS and then blocked with 5% normal donkey serum (Jackson Immunoresearch, West Grove, PA) in PBS for one hour at room temperature. Coverslips were then incubated with primary antibodies (see Supplementary Table 1) overnight at 4°C, rinsed and incubated with combinations of AlexaFluor 488- and AlexaFluor 594-conjugated anti-rabbit and anti-mouse IgGs (Life Technologies; 1:500) for 60 minutes at room temperature. The cells were counterstained with Hoechst 33258 (1 μg/mL; Life Technologies) for 10 minutes at room temperature and mounted onto glass microscope slides with fluorescent mounting medium (DAKO, Carpenteria, CA). Cells were imaged using a DMRXA2 epifluorescence microscope (Leica Microsystems) with an ORCA-ER cooled CCD camera (Hamamatsu, Hamamatsu City, Japan) and OpenLab 5 software (Improvision Ltd, Lexington, MA).

Images from 5 representative fields (392 μm × 512 μm) were collected from each coverslip using a 20× objective. After the first field was selected, non-overlapping images were captured above, below, to the left and to the right of the first field. The non-pyknotic cells were counted using ImageJ (National Institutes of Health, Bethesda, MD). The cell counts were averaged for each coverslip. Relative viability was defined as the percentage of non-pyknotic cells in response to TOP1 inhibitor treatment relative to DMSO-treated cells.

RNA Isolation

Primary cultures at DIV2 were treated with 1 μM CPT, 4 μM β-Lap, 1 μM ActD or DMSO for 2 hours. Total RNA was isolated from rat embryonic primary MNs, CNs and fibroblasts using the RNeasy Mini kit (QIAGEN, Valencia, CA) according to the manufacturer's directions. RNA integrity was assessed using the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA).

Microarray Analysis

Targets were prepared from 200 ng RNA using the Applause WT-Amp Plus ST System with the Encore Biotin Module (NuGEN, Inc., San Carlos, CA) following the manufacturer's protocol. Samples were hybridized to GeneChip Rat Gene 1.0ST Arrays (Affymetrix). These arrays were washed and stained in a GeneChip Fluidic Station 450 (Affymetrix) and then scanned with the GeneChip scanner 3000 7G (Affymetrix) following the manufacturer's protocols. The raw data were deposited into the NCBI Gene Expression Omnibus (GEO) under the accession number GSE67146.

Microarray Data Analysis

Expression values were calculated and LIMMA (linear models for microarray analysis) was performed as described previously [10]. The rank product non-parametric method RankProd was also used [11]. A false discovery rate (FDR) and an estimated percentage false positive (PFP) value less than 0.05 were considered significant. Identification of biological pathways and upstream regulators in rat MNs affected by CPT was completed using Ingenuity

Pathway Analysis (IPA version 21901358; QIAGEN Redwood City, Inc., Redwood City, CA) as described previously [12].

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR)

qRT-PCR was performed on drug treated samples as described previously [12] except that *glyceraldehyde 3-phosphate dehydrogenase* (*Gapd*), *ribosomal protein L13a* (*Rpl13a*) and *β-actin* (*Actb*) were used as reference transcripts (see Supplementary Table 2 for primer sequences).

Statistical Analysis

All quantitative data are expressed as means ± standard errors. Cell viability data were analyzed by ANOVA with a Bonferroni *post hoc* test. The qRT-PCR data were analyzed with T-tests. Statistical significance was set at $p \leq 0.05$. Statistical analyses of the quantitative data were completed using SPSS v.22.

RESULTS

Differential Responses of Various Cell Types to TOP1 Poisons and Inhibitors

We examined the effect of 3 TOP1 inhibitors—CPT, β-Lap and ActD—on the viability of CNs and MNs as well as fibroblasts. Immature (DIV2) and more mature (DIV5) cells were treated with TOP1 inhibitors for 24 hours. Cell viabilities were expressed relative to DMSO-treated cells. At DIV2, both CNs and MNs were more sensitive to CPT compared to fibroblasts (Figures 1A and 1D). With time in culture, fibroblasts became sensitive to the toxic effects of CPT. In contrast, the sensitivities of CNs and MNs to CPT did not markedly change over time. All 3 cell types were equally susceptible to β-Lap-induced toxicity at both time points but they were more resistant to β-Lap toxicity at DIV5 than DIV2 (Figures 1B and 1E). At DIV2, ActD yielded similar toxic effects in all cell types examined (Figure 1C); however, MNs at DIV5 were more sensitive to ActD toxicity than either CNs or fibroblasts (Figure 1F). Taken together, neurons were more sensitive to the toxic effects of TOP1 poisons than fibroblasts.

Differential Expression of *Top1* mRNAs and CPT Sensitivity

MNs and CNs may be more sensitive to TOP1 poison-induced toxicity because these cells may contain more TOP1 than other cell types. We compared basal *Top1* mRNA levels between MNs, CNs and fibroblasts using qPCR. *Top1* transcript levels were 3.8- and 4.5-fold higher in CNs and MNs, respectively, than in fibroblasts (Figure 2A). Transient treatment with 1 μM CPT for 2 hours resulted in reduced *Top1* mRNA levels in all 3 cell types; the magnitude of change was higher in CNs and MNs than in fibroblasts (Figure 2B). Additionally, treatment of MNs with β-Lap and ActD for 2 hours reduced *Top1* mRNA levels (Figure 2C).

Identification of Differentially Expressed RNAs in Response to CPT Treatment in MNs

To identify RNA transcripts whose levels are affected by CPT, DIV2 rat primary MNs were treated with either 1 μM CPT or DMSO (n = 3/group) for 2 hours. This concentration was

selected as it was the LC_{50} in rat primary MNs (1.1 ± 0.2 μM). Since there was minimal DNA damage and no cell death in treated MNs at this time point, cell death pathways should not be over-represented in the analysis of differentially expressed transcripts at this early time point. Differentially expressed transcripts were identified from the resultant microarray data using LIMMA and RankProd. 926 gene IDs (664 downregulated and 262 upregulated) were identified by both LIMMA and RankProd as being significantly different between CPT- and vehicle-treated MNs. Ingenuity Pathway Analysis (IPA) mapped 594 identified transcripts (142 upregulated and 452 downregulated; Supplementary Table 3) whose levels were altered by CPT, after manual removal of redundant probe set IDs.

To assess the biological relevance of the differentially expressed transcripts in CPT-treated MNs, we used the IPA Ingenuity Knowledge Base repository of literature-based biological data and its Upstream Regulator Analysis (URA) feature to identify overrepresented signaling pathways [13]. Given that there was a large number of canonical pathways overrepresented (Supplementary Table 4), analysis of these canonical pathways along with URA (Supplementary Table 5) suggest that MAP kinase and JNK signal transduction pathways are activated in MNs in response to CPT.

Validation of Differentially Expressed Transcripts Identified by Microarray Analysis

We used qRT-PCR to validate the changes in the mRNA levels of transcripts in MNs identified by microarray analysis in response to CPT using biological replicates. 4 transcripts—*FBJ murine osteosarcinoma viral oncogene homolog* (*Fos*), *early growth response 1* (*Egr1*), *dual-specificity tyrosine-phosphorylation regulated kinase 3* (*Dyrk3*) and *growth arrest and DNA-damage-inducible β* (*Gadd45b*)—were selected because they exhibited strong induction in response to CPT treatment and, in some cases, have been previously identified as being upregulated in response to CPT [14–16]. Exposure to CPT for 2 hours increased the mRNA levels of *Fos*, *Egr2*, *Dyrk3* and *Gadd45b* in MNs (Figure 3A).

Are the changes in these transcripts the specific result of CPT treatment or a generalized consequence of TOP1 inhibition? The changes in the levels of *Fos*, *Egr2*, *Dyrk3* and *Gadd45b* mRNAs were measured in MNs treated with β-Lap (4 μM) or ActD (1 μM) for 2 hours. These changes were compared to those elicited by 1 μM CPT treatment. The inhibitor concentrations used here were close to the LC_{50}s for each compound in MNs. The induction of all four mRNAs in response to CPT was smaller than the responses of MNs to ActD (Figure 3B). β-Lap increased *Egr2*, *Dyrk3* and *Gadd45b* mRNA levels in MNs more strongly than did CPT.

We next determined if the effects of CPT on target gene expression were unique to MNs. The levels of *Fos*, *Egr2*, *Dyrk3* and *Gadd45b* mRNAs were measured in CNs and fibroblasts treated with 1 μM CPT for 2 hours. *Fos* mRNA levels were increased in all 3 cell types (Figure 3C). Interestingly, the levels of *Egr2*, *Dyrk3* and *Gadd45b* mRNAs were elevated in CPT-treated CNs—as in treated MNs—but were reduced in CPT-treated fibroblasts (Figures 3D–F).

DISCUSSION

We showed in this study that CNs and MNs were more susceptible to the toxic effects of CPT than fibroblasts. This is consistent with other findings that exposure of CNs, MNs and cerebellar granule cell (CGC) neurons to CPT leads to apoptotic death [17–20]. Why are MNs and CNs more susceptible than fibroblasts to the toxic effects of TOP1 poisons? The abundance of TOP1 in these cells may explain the differential sensitivity to CPT and ActD. Short hairpin RNA (shRNA)-mediated suppression of *TOP1* expression in lymphoma cells confers resistance to CPT toxicity [21]. TOP1 is expressed in the central nervous system, especially in inhibitory neurons [22]. We show here that *Top1* mRNA levels were higher in cultured CNs and MNs, which were more sensitive to CPT and ActD toxicity, than in fibroblasts. Treatment of MNs, CNs and fibroblasts with CPT resulted in a rapid (2 hours) decline in *Top1* mRNA levels. Those cell types with higher basal *Top1* levels showed a sharper decline in *Top1* expression in response to CPT suggesting that the increased susceptibility of CNs and MNs to CPT may be independent of TOP1 expression.

CPT reduces the transcription of genes into mRNAs by RNA polymerase II [23]. mRNAs transcribed from long genes are downregulated in response to CPT in cancer cell lines, fibroblasts and in neurons while those from short genes show increased levels [24–26]. Some of these long transcripts suppressed by CPT are linked to autism [25]. In CNs, many of these transcripts suppressed by CPT encode synaptic proteins and, as a result, excitatory and inhibitory neurotransmission is reversibility blocked by CPT [27]. These observations along with our study collectively highlight the importance of TOP1 in transcriptional regulation in neurons and could explain why they are more sensitive to TOP1 poisons like CPT and ActD.

To delineate the early mechanisms of the neuronal response to CPT, we identified differentially expressed transcripts in CPT-treated MNs using microarrays. DNA damage response and cell death pathways were not overrepresented in the differentially expressed transcripts of MNs treated with CPT for 2 hours. Many of these differentially expressed transcripts are classified as immediate early genes (IEGs) in that their expression is rapidly regulated in response to stimulus or injury [28]. CPT rapidly induces the transcription of IEGs like *Fos*, *Jun* and *Egr1* in non-neuronal cells [14–16] as well as in neurons (*this study*). The IEGs *Fos* and *Jun* were upregulated in response to DNA damage induced by CPT and likely mediated their downstream effects through activation of ERK and JNK MAP kinases. ERK and JNK activities are increased in CNs shortly after treatment with CPT [18;29]. URA of upregulated transcripts revealed early activation of MAPK and JNK pathways in MNs in response to CPT.

DYRK3 is a member of a family of dual-specificity tyrosine-related protein kinases that autophosphorylate on tyrosine residues as well as phosphorylate serine and threonine amino acids on other proteins [30]. In this study, we have shown that *Dyrk3* mRNA levels were upregulated in neurons in response to CPT treatment. DYRKs are implicated in many intracellular processes including cell survival/death, differentiation and gene expression [31]. DYRKs differentially regulate the activity of certain substrates like p53, a key mediator

in the DNA damage response [32–34]. Future studies will delineate the role of DYRK3 in the response of neurons to TOP1 poisons as well as other forms of injury.

In summary, we have identified transcripts like *Egr2*, *Gadd45b* and *Dyrk3* which are increased in neuronal cells, but not in non-neuronal fibroblasts, in response to transient CPT treatment. These early response transcripts could help explain the enhanced sensitivity of neurons to TOP1 poisons. These differentially upregulated transcripts could become targets for the development of therapeutic strategies which minimize neuronal damage in response to chemotherapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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HIGHLIGHTS

- Cultured neurons are more sensitive than fibroblasts to camptothecin and actinomycin D toxicity
- *Topoisomerase I* mRNA levels are higher in neurons than in fibroblasts
- Camptothecin increases *Egr-1*, *Gadd45b* and *Dyrk3* in neurons but not fibroblasts

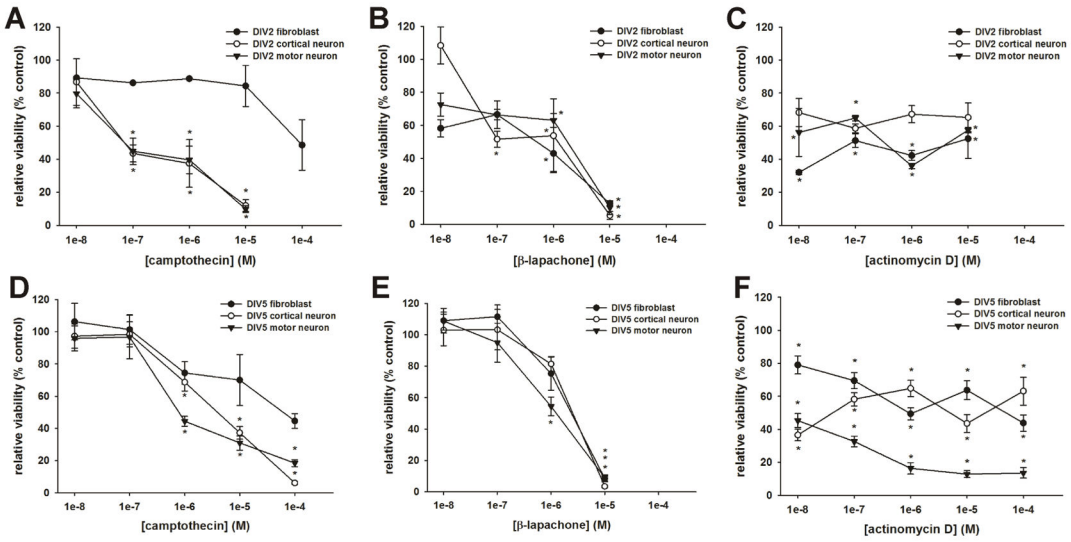

Figure 1. Effects of TOP1 inhibitors on the viability of rat embryonic primary MNs, CNs and fibroblasts

Primary cultures of rat MNs (closed triangles), CNs (open circles) and fibroblasts (closed circles) at DIV2 **(A–C)** or DIV5 **(D–F)** were treated with different concentrations of CPT **(A, D)**, β-Lap **(B, E)** or ActD **(C, F)** for 24 hours (n = 3/treatment). After treatment, the number of viable cells was counted for each condition. Cell viabilities were expressed relative to DMSO-treated cells. The asterisk (*) denotes a statistically significant difference ($p \leq 0.05$) relative to DMSO-treated cells.

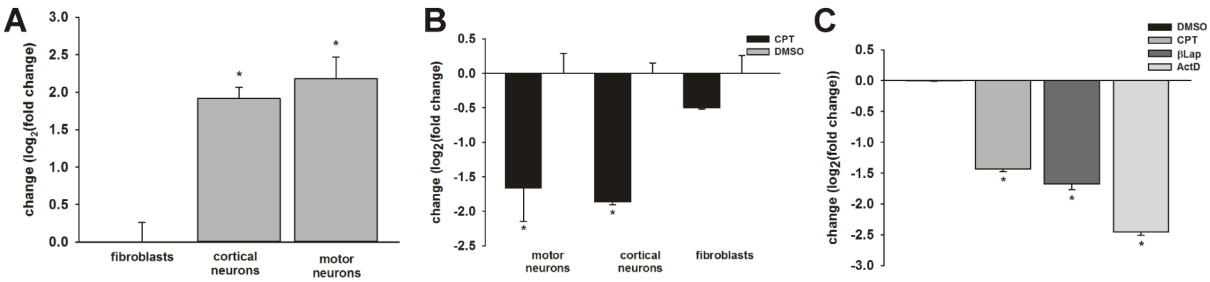

Figure 2. Effects of TOP1 inhibitors on *Top1* mRNA expression in rat embryonic primary cultures

The levels of *Top1* mRNA were measured using qRT-PCR in cells treated with drugs (n = 3/treatment). **(A)** The relative amounts of *Top1* mRNA in rat embryonic primary CN and MN cultures relative to *Top1* mRNA levels in rat fibroblasts. The magnitude of change is expressed as $\log_2$(fold change). The asterisk (*) denotes a statistically significant difference ($p \leq 0.05$) relative to the magnitude of change in fibroblasts. **(B)** The magnitude of change in *Top1* mRNA levels in CPT-treated MNs, CNs or fibroblasts relative to DMSO-treated cells. The asterisk (*) denotes a statistically significant difference ($p \leq 0.05$) relative to DMSO-treated cells. **(C)** The magnitude of change in *Top1* mRNA levels in rat MNs treated with CPT (grey bar), β-Lap (dark grey bar) or ActD (light grey bar) relative to DMSO-treated (black bar) MNs. The asterisk (*) denotes a statistically significant difference ($p \leq 0.05$) relative to DMSO-treated MNs.

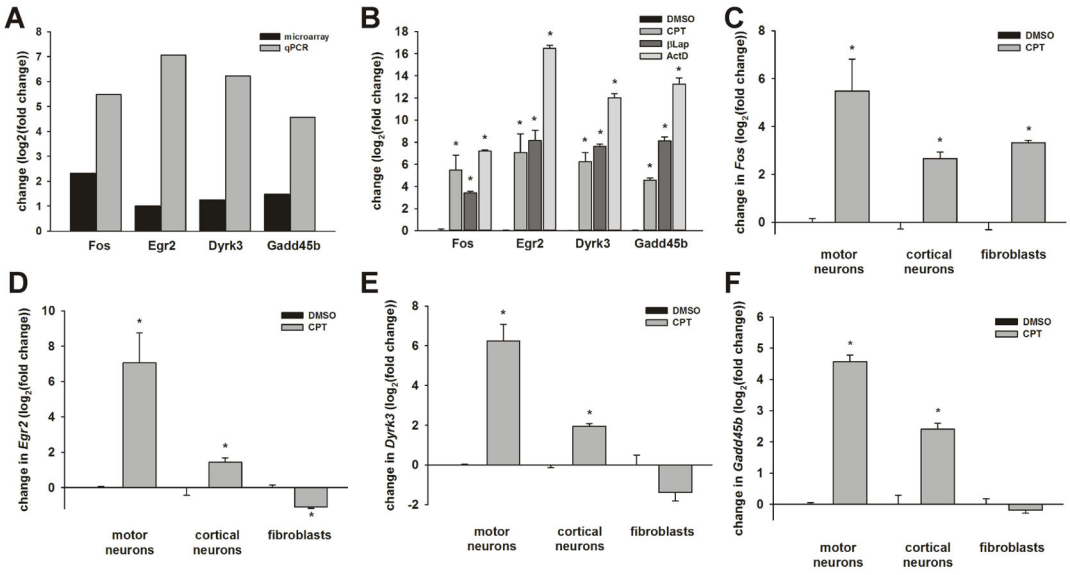

Figure 3. Expression of differentiated expressed transcripts identified by microarray in TOP1 poison-treated cells

The levels of *Fos*, *Egr2*, *Dyrk3* and *Gadd45b* transcripts were measured using qRT-PCR in cells treated with drugs (n = 3/treatment). **(A)** The magnitude of change ($\log_2$(fold change)) of target transcripts in CPT-treated rat MNs as determined by microarray (black bars) or qRT-PCR (grey bars). **(B)** The magnitude of change of target transcripts in rat MNs treated with CPT (black bars), β-Lap (light grey bars) or ActD (dark grey bars) relative to DMSO-treated MNs. The magnitudes of change of *Fos* **(C)**, *Egr2* **(D)**, *Dyrk3* **(E)** and *Gadd45b* **(F)** in CPT-treated MNs, CNs or fibroblasts relative to DMSO-treated cells. The asterisk (*) denotes a statistically significant difference ($p \leq 0.05$) relative to DMSO-treated cells.