Final Project Report

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"Apoptosis in cultured brain cells following exposure to radiofrequency radiation"

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Introduction

The rapid adoption of mobile phone use by the public has had very real benefits; however, concerns and fears relating to potential health risks associated with mobile phone use are widespread. Exposure guidelines are based upon well established thermal effects but the potential effects of low level exposures remain an area of controversy and uncertainty. Two key areas of public concern relate to the possible effects of mobile phone use on brain function and on the development of brain cancer. Despite conflicting findings, these concerns have been raised by epidemiological studies suggesting that long term or use of a mobile phone associates with the occurrence of acoustic neuroma (Lönn *et al* 2004; Mild *et al* 2007; Hours *et al* 2007).

Insufficient energy is delivered by radiofrequency (RF) fields to cause direct DNA damage. Thus RF fields are unlikely to be initiators of tumorigenesis, however less certainty is associated with potential promoting effects. Such promoting effects would likely be associated with proliferation of abnormal cells as a consequence of induced cell death. Brain injury, for example as a result of cerebral ischemia, is associated with neuronal apoptosis – a process of active cell death - (Linnik *et al* 1995). Apoptosis also plays a role in the process of carcinogenesis (Zoring *et al* 2001).

Given the involvement of apoptosis in brain disease and the sensitivity of the process to cellular insult, the aim of this study is to investigate whether RF fields characteristic of mobile phones can induce apoptosis in neuroblastoma cells in proliferating and differentiated states, by using three independent assays for apoptosis following a time course 0 to 48 hours post exposure. In addition, alterations in the expression of three of the genes that regulate apoptosis, *p53*, *c-jun* and *bag-1*, following RF exposure were studied.

Methods

RF exposures were carried out in a sXc900 exposure system based on two identical rectangular waveguide cavities, (Information Technologies in Society Foundation (IT'IS), Zurich), installed in tissue culture incubators at 37°C with humidified atmospheres of 5% CO_2 in air. A specially designed holder allows eight 35mm Petri dishes to be placed accurately inside each chamber. Good exposure and environmental control is achieved using field sensors, temperature sensors for the air environment and an optimised airflow system. A computer controlled signal unit allows the application of various GSM modulated and non-modulated signals. Field strengths, temperature and fan currents as well as all commands are continuously logged to encrypted files. The assignment of either waveguide to be the sham or active is made randomly by the controlling computer and the coding for this is held at IT'IS until broken when the assay data are complete. Full details of the apparatus, the signal profile, the exposure geometry and dosimetry is described by Schuderer *et al* (2004).

Proliferating murine neuroblastoma Neuro2a (N2a) cells were seeded at a density of 2 x 10^5 cells into 35 mm Petri dishes with 3ml of growth medium 24 hours before being placed into the RF exposure system. N2a cells were induced to enter a post-mitotic state and differentiate into neurone-like cells by serum withdrawal. Briefly, cells were maintained at confluence for at least 3 days before seeding them at a density of 2 x 10^5 cells into 35 mm Petri dishes with 3ml of growth medium containing only 1% serum, 24 hours before being placed into the RF

exposure system. One hour before the RF exposure, Petri dishes containing the proliferating or differentiated N2a cells were placed inside the waveguides to allow the temperature to stabilise. Cells were exposed or sham exposed for 24 hours to a 935 MHz signal at an SAR of 2 W/kg. Three signal types were used, GSM basic, GSM talk and a continuous wave (CW) unmodulated signal comprising just the carrier frequency. The measured increase in temperature of the cells due to the RF radiation was around $0.06^{o}C$, which is considered sufficiently low that any effect observed could be attributed to non-thermal processes.

Cells irradiated with 4.0 Gy of 250 kVp x-rays at a dose rate of 1.0 Gy/min were used as a positive control together with a zero dose control. The irradiations were carried out at room temperature and the samples were also maintained at room temperature whilst being conveyed between the x-ray facility and laboratory. Following the x-ray, RF radiation, and control exposures the dishes were held in an incubator and assessed for the presence of cells undergoing apoptosis at several time points. Each exposure regime was repeated three times.

Three independent assays for apoptosis were employed using commercially available kits; the annexin V-FITC detection kit (BD Pharmingen), CaspaTag pan-caspase *in situ* assay kit (Chemicon International) and the *in situ* end labelling Apo-Direct kit (BD Pharmingen). The kits were used according to the manufacturer's protocols and for each exposure regime / time point 1000 cells were analysed under a fluorescence microscope for the annexin V and caspase assays, while 3000 cells were scored using the Apo direct assay. Each exposure regime / time point was replicated three times. The three assays detect cellular changes at different stages in the apoptosis pathway. The annexin V binding assay detects an early event and the *in situ* end labelling assay detects DNA fragmentation, which is typical of late-stage apoptotic cells. The caspase activation assay detects the cascade of proteolytic enzymes called caspases; a central feature of the apoptotic process. Therefore it would be expected for the annexin V assay to show an elevated response sooner than the caspase activation assay, which in turn would so an increased response sooner than the *in situ* end labelling (Apo-Direct) assay for the positive controls using x-rays.

N2a cells for gene expression analysis were collected from four 35 mm Petri dishes each for the exposed and control samples and stored at -70ºC in RNA*later* (Sigma). RNA was extracted using the RNAqueous-4PCR Kit (Ambion) and the assessment of gene expression was made using the TaqMan (Trade mark – Applied Biosystems) assay system. The TaqMan system is based on *Taq* polymerase 5'-3' nuclease activity, which cleaves a dually labelled non-extendible TaqMan probe designed to hybridize to a sequence between the forward and the reverse primer for every particular amplicon. The probe has a quencher dye on its 3' end and a reporter dye at the 5' end. Fluorescence emission from the reporter is quenched by the quencher dye until nuclease degradation during the extension phase of the PCR separates the two dyes and enables detection of the reporter dye fluorescence. Prevalidated Primers and probes were purchased from Applied Biosystems. mRNA was used as a template for the production of first-strand cDNA using random primers (Taqman Reverse Transcription reagents, N8080234, Applied Biosystems). The cDNA preparation was used as the template for each TaqMan assay for the p53, c-jun, bag-1 and the reference GAPDH genes. No-reverse transcriptase was used for the control of RT reaction. The same preparations of primers and probes were used in all experiments. Reporter dyes and quencher dyes for all probes were 6-carboxyfluorescein (FAM) and 6-carboxytetramethylrhodamine (TAMRA), respectively. The TaqMan PCR reaction conditions were: 2 min at 50 °C, 10 min at 95 °C, then 40 cycles each of 15 s at 95 °C and 1 min at 60 °C on Optical 96-well plates, covered with Optical caps. Each plate contained triplicates of the test cDNA templates, and no-template controls for each reaction mix. All samples contained TaqMan master mix (Applied Biosystems) and TaqMan probe. During the assay, the linear increase in fluorescence signals from the reporter dye was recorded by an ABI Prism 7500.

Results and Discussion

The results of the apoptosis assays are shown in Figures 1 – 6. All the data points are total values ± standard error from three pooled experiments. Chi squared tests for homogeneity were carried out as well as Student's t-tests to determine the significance of any difference

between the control and exposed cells. A *p* value of less than 0.05 was considered significant.

When the results were decoded it was evident with the positive x-ray control data that the expected time sequence of peaks in apoptosis levels were observed with the three assays. In Figure 1, which shows annexin V binding on proliferating cells, it can be seen in the first column of each time set an x-ray induced increase in apoptotic cells was observed, peaking at 2 hours post exposure. The level of apoptosis remained significantly higher than in the unirradiated controls (second column) between 4 and 8 hours post exposure. At 24 hours post exposure the level of apoptosis was not significant different from the unirradiated control, but at 48 hours the level of apoptosis significantly increased again. For the same assay, but using differentiated cells, Figure 2, a broad x-ray induced peak was observed between 2 and 8 hours post exposure, although the increase in the level of apoptosis was only significant at 4 hours (p = 0.04). The level of apoptosis returned to control levels at 24 hours and although rose again at 48 hours post exposure the rise was not significant. However, with the caspase activation assay and proliferating cells, Figure 3, there was a steady rise in apoptosis levels, which peaked at 24 hours post exposure and although this fell at 48 hours the level was still above that seen in the unirradiated samples. Using differentiated cells, Figure 4, the levels of apoptosis rose at 8 hours post exposure and remained at an elevated level at 24 and 48 hours. Figures 5 and 6 show that increased levels in apoptosis were observed using the *in situ* end labelling assay in both proliferating and differentiated cells exposed to 4 Gy x-rays at 24 and 48 hours. These results with the positive x-ray control therefore broadly conform with the time-peak sequences expected for the three assays.

In Figures 1 – 6 both proliferating and differentiated cells exposed to RF radiation show no obvious peaks in apoptosis with any of the assays. With one exception, for each signal and at all time points the level of apoptosis is not significantly different (p > 0.05) from its individually matched sham exposed control. The students t-test revealed one statistically significant difference (p=0.03) between the sham and RF GSM talk exposed proliferating cells at 8 hours using the caspase assay (shown in red in Figure 3). The exposed cells showed a decrease in the level of apoptosis compared to the sham, but on repetition of this exposure regime no significant difference was found (data not shown). The lack of significant changes in apoptosis for this signal / time in the other assays also serve to suggest it is a chance finding.

These results are in agreement with the earlier studies that investigated the induction of apoptosis in non-dividing human lymphocytes exposed to GSM modulated RF radiation (Capri *et al* 2004a, b), together with studies using cell lines derived from human peripheral blood (Hook *et al* 2004; Lantow *et al* 2006). However, Marinelli et al (2004) using a human leukaemia cell line observed an increase in the cells undergoing apoptosis after a 2 hour exposure to a 900MHz CW signal that decreased after 24 / 48 hours. More recent studies have used human neuroblastoma cells to assess levels of apoptosis after exposure to GSM modulated RF radiation (Gurisik *et al* 2006, Merola *et al* 2006 and Joubert *et al* 2006). In these studies, as with the present work, the authors also concluded that under athermal conditions, exposure to RF radiation did not induce alterations in the levels of apoptosis.

In two recent studies Joubert *et al* (2007 and 2008) have exposed primary cortical neurones to RF fields for 24 hours and used several methods to detect levels apoptosis. A GSM modulated 900 MHz signal was used in the first study (Joubert *et al* 2007) and no difference in apoptosis rates between the exposed and non exposed cells were found. This is in agreement with a study by Lagroye *et al* (2002) using primary neuronal cells and astrocytes. However, in a second study (Joubert *et al* 2008) a significant difference in apoptosis rate between exposed and sham exposed cells was found with a 900 MHz CW signal, but the exposure was not athermal. During the exposure a temperature rise of 2ºC was noted and although control experiments with neurons exposed to 37ºC and 39ºC were also performed the apoptosis rate in the RF exposed cells was also significantly different from these controls.

Figures 7 – 9 show the measurements of the relative gene expression of *p53*, *c-jun* and *bag-1* in proliferating and differentiated N2a cells following a 24 hour exposure to a 935MHz GSM basic signal with a SAR of 2 W/kg at 2 and 24 hours post exposure. Student's t-tests showed no significant changes in gene expression associated with RF radiation, except for *c-jun* in

proliferating N2a cells assayed 2 hours post exposure (Figure 8). However, as the increase was small further repeat measurements would be needed to confirm the result. Overall the results are in agreement with a study by Hirose *et al* (2006) that found no significant difference in the expression levels of p53 or in the gene expression of subsequent downstream targets of p53 signalling in human glioblastoma cells exposed to 2.1425 GHz RF radiation for 24 hrs. However, Marinelli *et al* (2004) found p53 levels increased after a 2 hour exposure to a 900MHz CW signal that retuned to basal levels after 24 hours. By contrast, Ivaschuk *et al* (1997) using differentiated PC12 pheochromocytoma cells found no alteration in the expression of *c-jun* and *c-fos* after a 836MHz intermittent exposure.

Mouse embryonic stem cells selectively differentiated into neural progenitor cells were used by Nikolova *et al (2005)* to investigate the effects of RF radiation on proliferation, apoptosis and DNA damage, as well as the transcript levels of cell cycle regulatory, apoptosis related and neural specific genes and proteins. Cells were exposed to an intermittent 1.71 GHz GSM signal 6 and 48 hours. Although changes in mRNA levels were detected for some cell cycle regulatory and apoptosis related genes, no effect of RF radiation was detected on nuclear apoptosis, cell proliferation and chromosome aberrations. The authors postulated that any RF radiation effect at the mRNA level does not lead to detectable changes in cell physiology.

Conclusion

In this study exposure of mouse neuroblastoma cells, either in a proliferating or a differentiated state, to a GSM basic, talk or continuous wave signal of 935MHz for 24 hours at a SAR of 2 W/kg did not induce consistent significant differences in the levels of apoptosis compared to sham exposed / control samples; as measured by the annexin V, the caspase activation and the Apo direct assays between 0 and 48 hours post exposure. In addition no major impact of exposure to a 935MHz GSM basic signal for 2 or 24 hours on the expression of the *p53*, *c-jun* and *bag-1* genes was detected. The data presented here serve to strengthen the case that mobile phone characteristic RF exposures do not significantly affect apoptosis or apoptotic signalling in brain cells.

References

Capri M., Scarcella E., Fumelli C., Bianchi E., Salvioli S., Mesirca P., Agostini C., Antolini A., Schiavona A., Castellani G., Bersani F. and Franceschi C. 2004 (a). In vitro exposure of human lymphocytes to 900 MHz CW and GSM modulated radiofrequency: Studies of proliferation, apoptosis and mitochondrial membrane potential. Radiat. Res.162: 211-218.

Capri M., Scarcella E., Bianchi E., Fumelli C., Salvioli S., Mesirca P., Agostini C., Remondini D., Schuderer J., Kuster N., Franceschi C. and Bersani F. 2004 (b).1800 MHz radiofrequency (mobile phones, different Global System for Mobile communication modulations) does not affect apoptosis and heat shock protein 70 level in peripheral blood mononuclear cells from young and old donors. Int. J. Radiat. Biol. 80: 389-397.

Gurisik E., Warton K., Martin D.K. and Valenzuela S.M. 2006. An in vitro study of the effects of exposure to a GSM signal in two human cell lines : monocytic U937 and neuroblastoma SK-N-SH. Cell Biol. Int. 10: 793-799,

Hirose H., Sakuma N., Kaji N., Suhara T., Sekijima M., Nojima T and Miyakoshi J. 2006. Phosphorylation and gene expression of p53 are not affected in human cells exposed to 2.1424 GHz band CW or W-CDMA modulated radiation allocated to mobile radio base stations. Bioelectromagnetics. 27: 494-504.

Hook G.J., Zhang P., Lagroye I., Li L., Higashikubo R., Moros E.G., Straube W.L., Pickard W.F., Baty J.D. and Roti-Roti J.L. 2004. Measurement of DNA damage and apoptosis in Molt-4 cells after in vitro exposure to radiofrequency radiation. Radiat. Res.161: 193-200.

Hours M., Bernard M., Montestrucq L., Arslan M., Bergeret A., Deltour I. and Cardis E. 2007. Cell phones and risk of brain and acoustic nerve tumours: the French INTERPHONE case-control study. Rev.Epidemiol. Sante Publique. 55(5): 321-332.

Ivaschuk O.I., Jones R.A., Ishida-Jones T., Haggren w., Adey W.R. and Phillips J.L. 1997. Exposure of nerve growth factor-treated PC12 rat pheochromocytoma cells to a modulated radiofrequency field at 836.55 MHz: Effects on c-jun and c-fos expression. Bioelectromagnetics. 18: 223-229.

Joubert V., Leveque P., Rametti A., Collin A., Bourthoumieu S. and Yardin C. 2006. Microwave exposure of neuronal cells in vitro: Study of apoptosis. Int. J. Radiat. Biol. 82: 267-275.

Joubert V., Leveque P., Cueille M., Bourthoumieu S. and Yardin C. 2007. No apoptosis is induced in rat cortical neurons exposed to GSM phone fields. Bioelectromagnetics. 28: 115-121.

Joubert V., Bourthoumieu S., Leveque P. and Yardin C. 2008. Apoptosis is induced by radiofrequency fields through the caspase-independent mitochondrial pathway in cortical neurones. Radiat. Red. 169(1): 38-45.

Lantow M, Viergutz T., Weiss G. and Simkó M. 2006. Comparative study of cell kinetics and induction of apoptosis or necrosis after exposure of human mono mac 6 cells to radiofrequency radiation. Radiat. Res. 166: 539-543.

Lagroye I., Bersani F., Agostini C., Bianchi E., Capri M., Dulou P.E., Fumelli C., Haro E., Mesirca P., Poulletier de Gannes F., Scarcella E and Veyret B. 2002. Do GSM-900 signals induce apoptosis in mammalian immune and nervous system cells? 2nd International Workshop on Biological Effects of EMF's, Rhodes Crète. 404-408.

Linnik M.D., Millar J.A., Sprinkle-Cavallo J., Mason P.J., Thompson F.Y., Montgomery L.R. and Schroeder K.K.1995. Apoptotic DNA fragmentation in the rat cerebral cortex induced by permanent middle cerebral occlusion. Mol. Brain Res. 32: 116-124.

Lönn S., Ahlbom A., Hall P. and Feychtung M. 2004. Mobile phone use and the risk of acoustic neuroma. Epidemiology. 15: 653-659.

Marinelli F., La Sala D., Cicciotti G., Cattini L., Trimarchi C., Putti S., Zamparelli A., Giullani L., Tomassetti G. and Cinti C. 2004. Exposure to 900 MHz electromagnetic field induces an unbalance between pro-apoptotic and pro survival signals in T-lymphoblastoid leukaemia CCRF-CEM cells. J. Cell. Physiol. 198: 324-332.

Merola P., Marino C., Lovisolo G.A., Pinto R., Laconi C. and Negroni A. 2006. Proliferation and apoptosis in a neuroblastoma cell line exposed to 900 MHz modulated radiofrequency field. Bioelectromagnetics. 27: 164-171.

Mild K.H., Hardell L., and Carlberg M. 2007. Pooled analysis of two Swedish case-control studies on the use of mobile and cordless telephones and the risk of brain tumours diagnosed during 1997-2003. Int.J. Occup. Saf. Ergon. 13(1): 63-71.

Nikolova T., Czyz J., Rolletschek A., Blyszczuk P., Fuchs J., Jovtchev G., Schuderer J., Kuster N. and Wobus A.M. 2005. Electromagnetic fields affect transcript levels of apoptosis-related genes in embryonic stem cell-derived neural progenitor cells. FASEB J. 10: 1686-

Schuderer J., Spät D., Samaras T., Gröbli J-C., Oesch W. and Kuster N. 2004. *In vitro* exposure systems for RF exposures at 900 MHz. IEEE Transactions on Microwave Theory and Techniques. 52: 2067-2075.

Zornig M., Hueber A., Baum W. and Evan G. 2001. Apoptotic regulators and their role in tumorigenesis. Biochim. Biophys. Acta. 1551: F1-37.
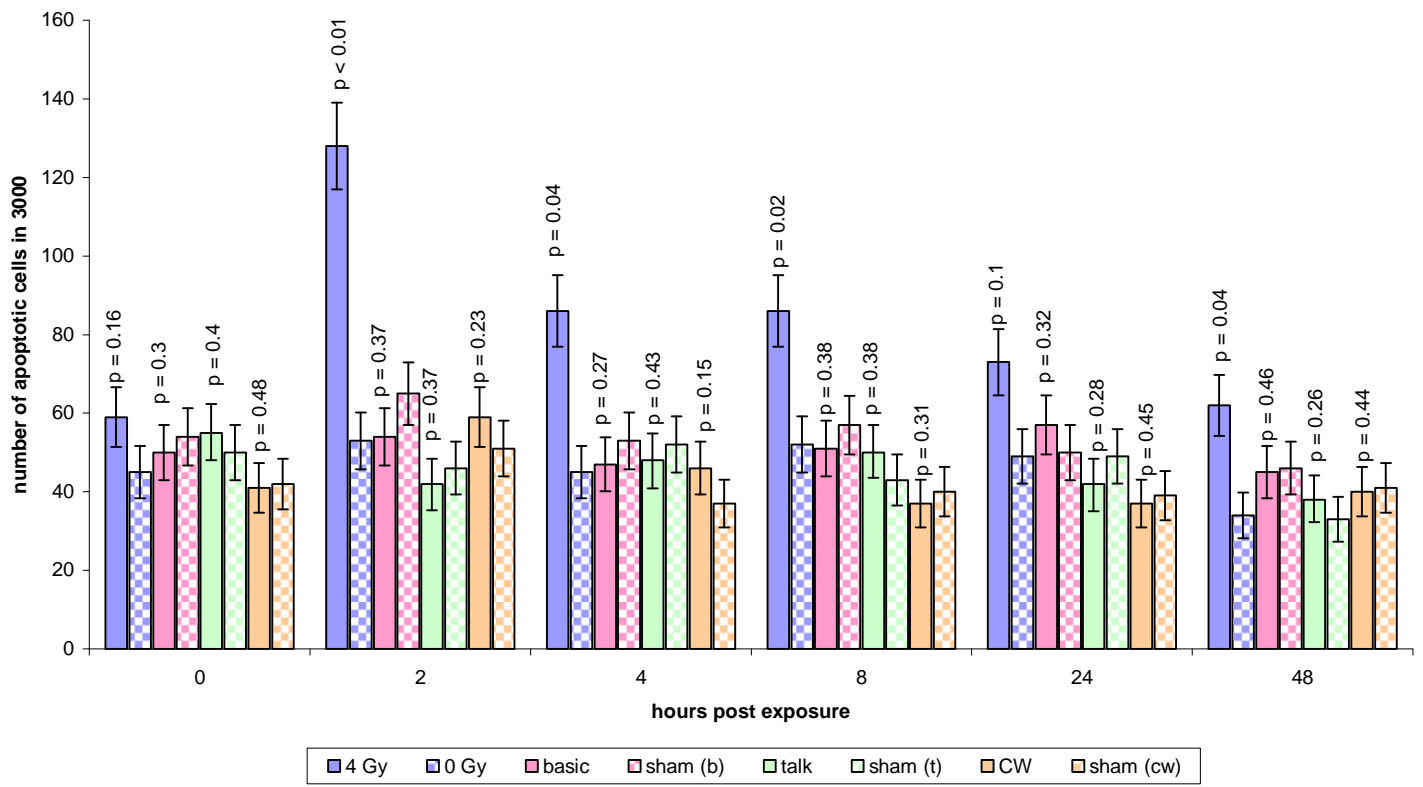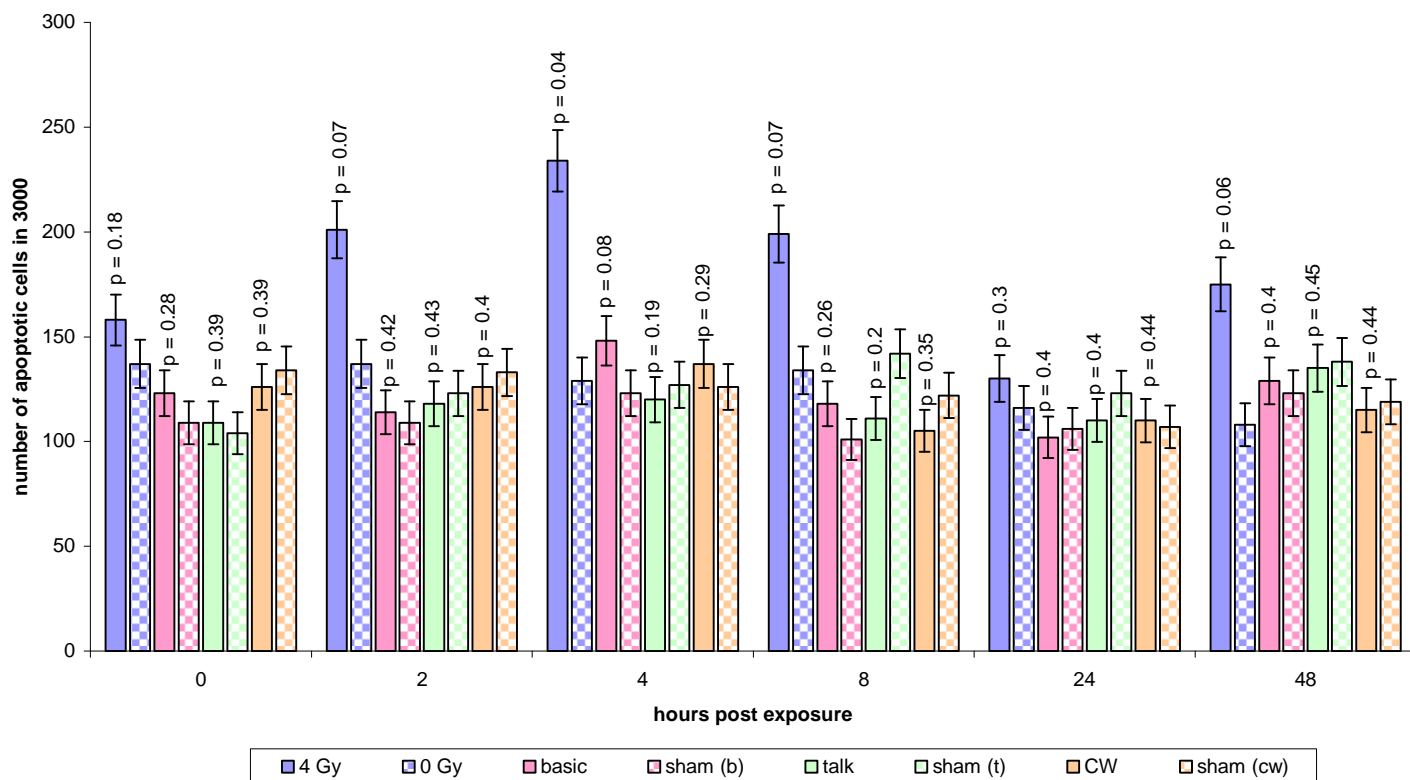
Figure 1. Measurement of apoptosis levels using the Annexin V binding assay in proliferating N2a cells after a 24 hour exposure to GSM basic, GSM talk or GSM CW signals (2 W/kg) / sham exposures, together with positive controls exposed to 4 Gy x-rays and unirradiated controls. Each data point shows the combined total ± SE of apoptotic cells from assaying 1000 cells in each of three repeated experiments. p values for each pair of exposed/sham data points are also shown above the exposed data point.
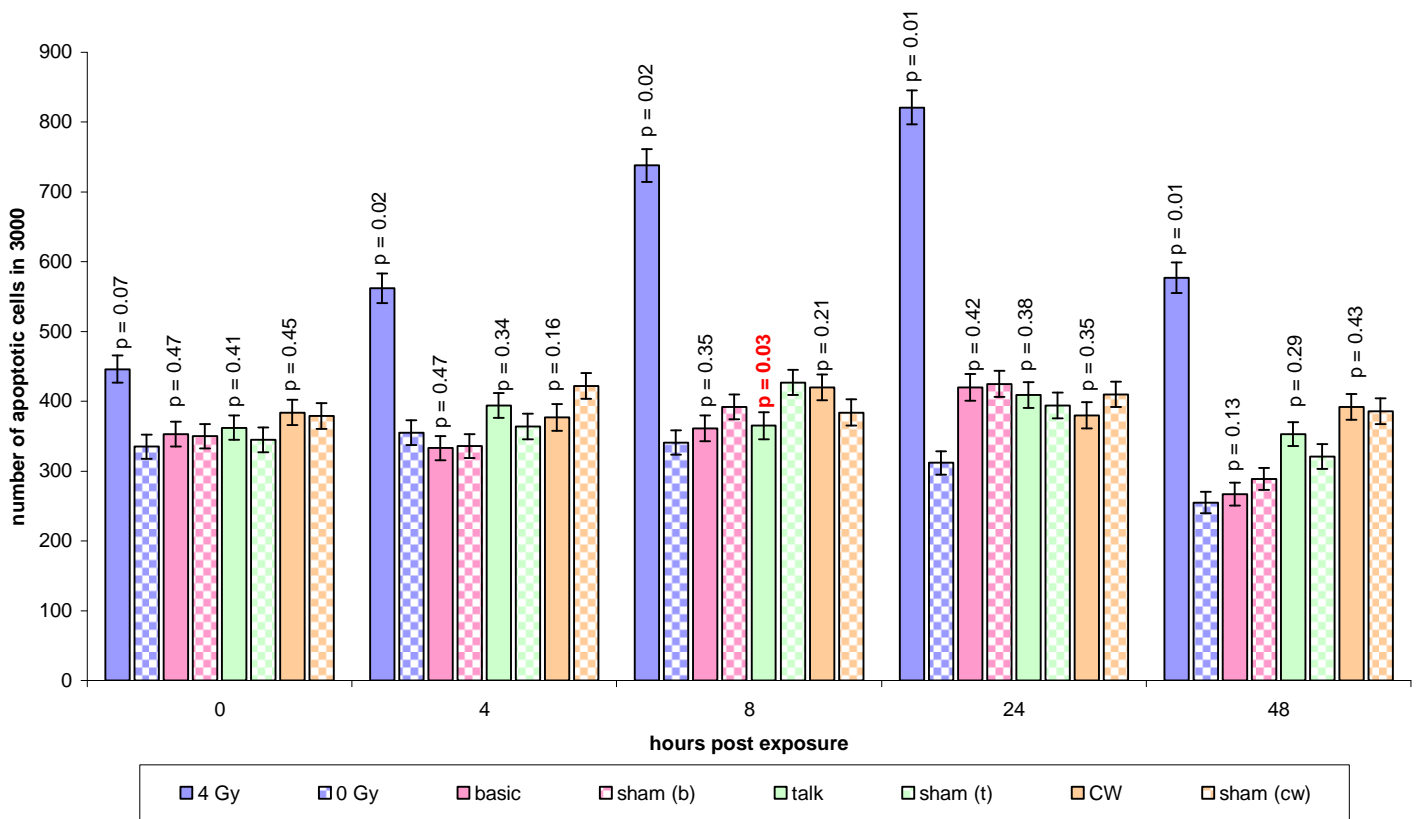
Figure 2. Measurement of apoptosis levels using the Annexin V binding assay in differentiated N2a cells after a 24 hour exposure to GSM basic, GSM talk or GSM CW signals (2 W/kg) / sham exposures, together with positive controls exposed to 4 Gy x-rays and unirradiated controls. Each data point shows the combined total ± SE of apoptotic cells from assaying 1000 cells in each of three repeated experiments. p values for each pair of exposed/sham data points are also shown above the exposed data point.
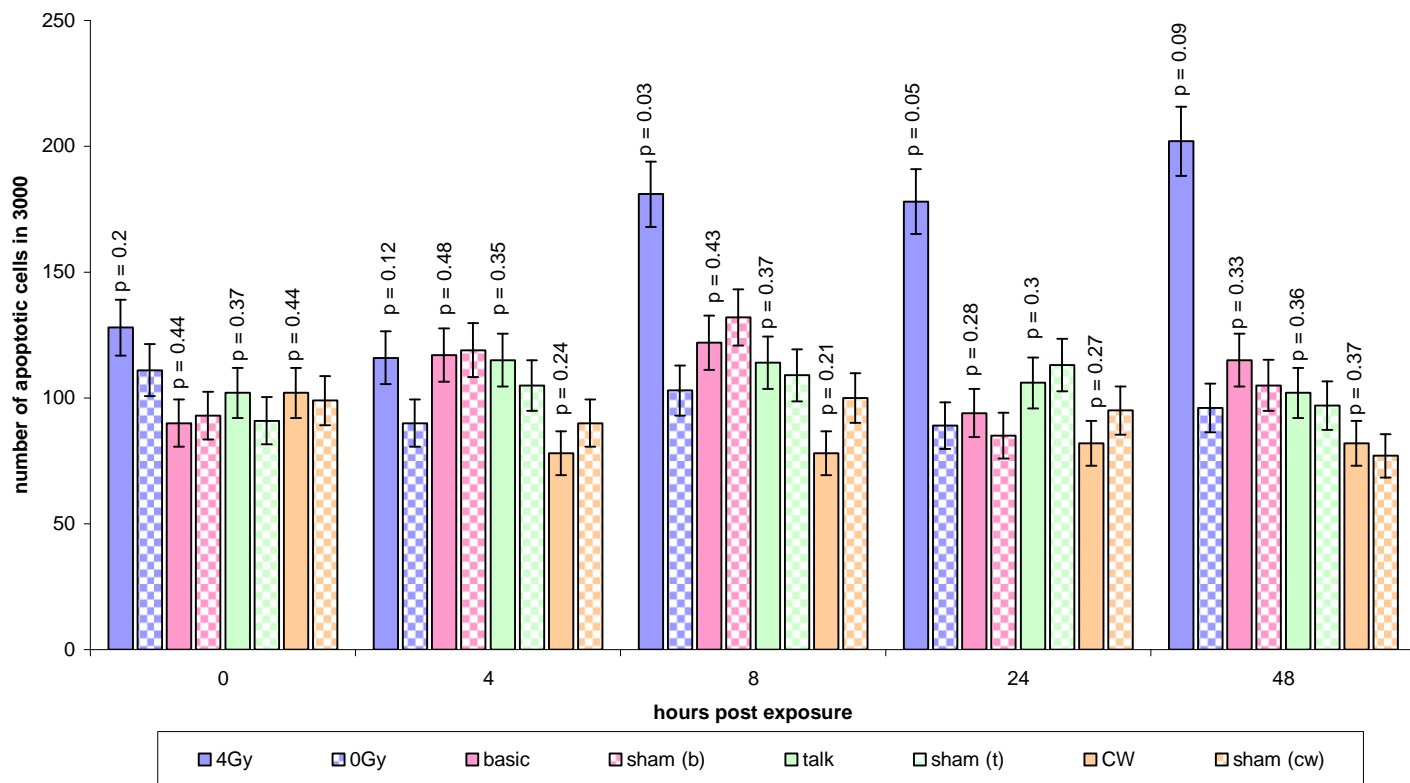
Figure 3. Measurement of apoptosis levels using the caspase activation assay in proliferating N2a cells after a 24 hour exposure to GSM basic, GSM talk or GSM CW signals (2 W/kg) / sham exposures, together with positive controls exposed to 4 Gy x-rays and unirradiated controls. Each data point shows the combined total ± SE of apoptotic cells from assaying 1000 cells in each of three repeated experiments. p values for each pair of exposed/sham data points are also shown above the exposed data point.
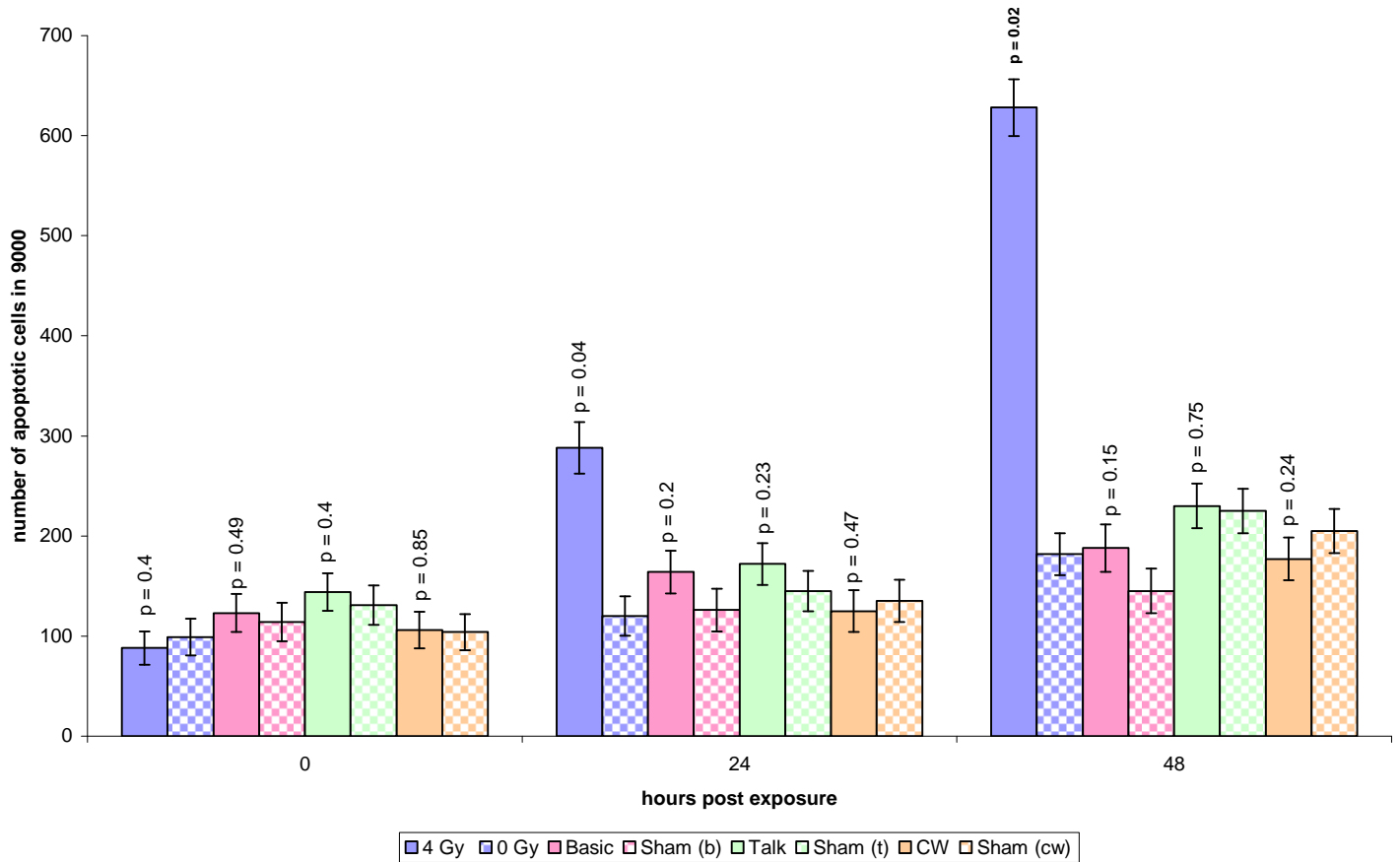
Figure 4. Measurement of apoptosis levels using the caspase activation assay in differentiated N2a cells after a 24 hour exposure to GSM basic, GSM talk or GSM CW signals (2 W/kg) / sham exposures, together with positive controls exposed to 4 Gy x-rays and unirradiated controls. Each data point shows the combined total ± SE of apoptotic cells from assaying 1000 cells in each of three repeated experiments. p values for each pair of exposed/sham data points are also shown above the exposed data point.

Figure 5. Measurement of apoptosis levels using the Apo-direct assay in proliferating N2a cells after a 24 hour exposure to GSM basic, GSM talk or GSM CW signals (2 W/kg) / sham exposures, together with positive controls exposed to 4 Gy x-rays and unirradiated controls. Data show as total values ± SE for three experiments of FITC$^+$ cells. p values for each pair of exposed/sham data points are also shown above the exposed data point.
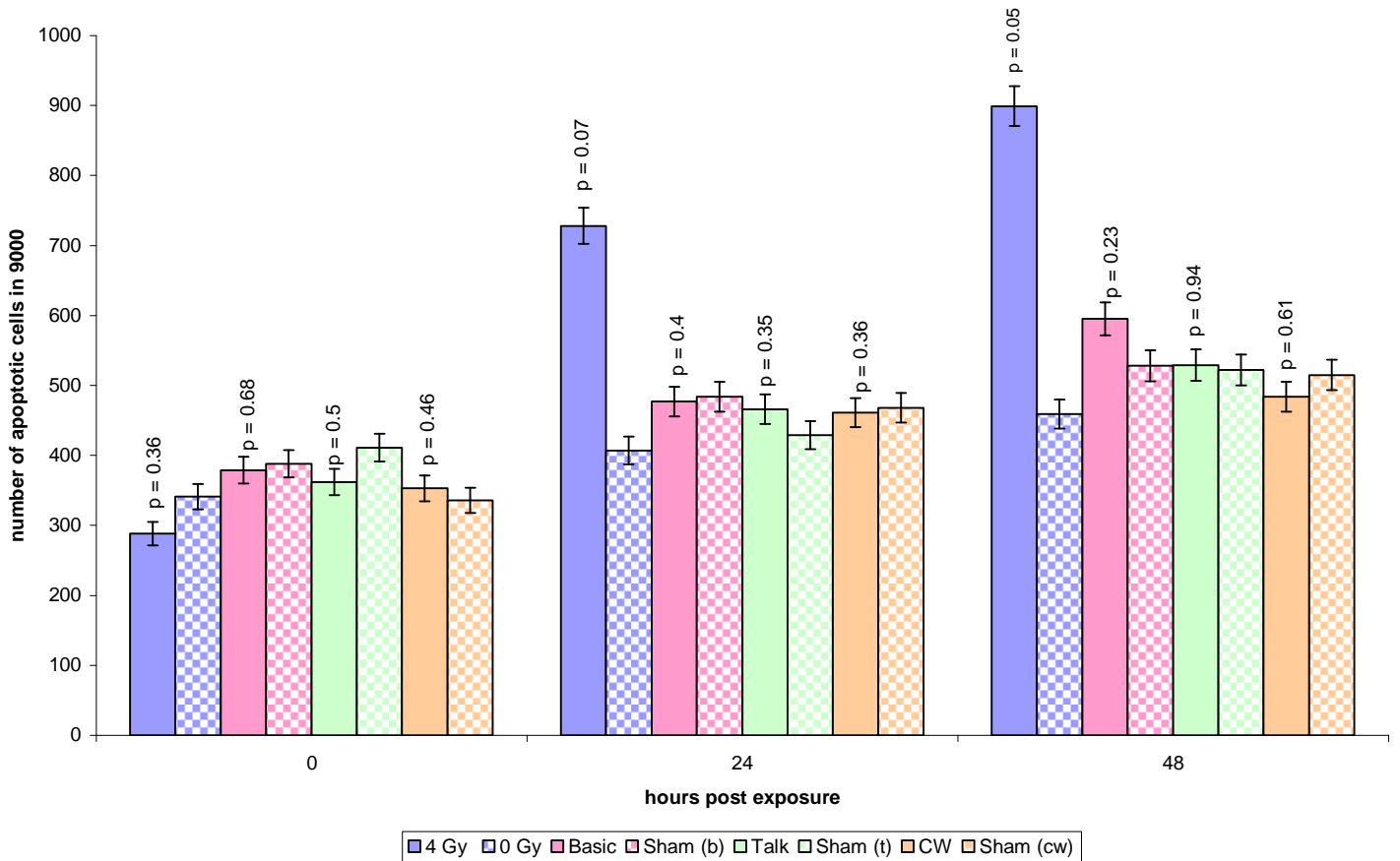
Figure 6. Measurement of apoptosis levels using the Apo-direct assay in differentiated N2a cells after a 24 hour exposure to GSM basic, GSM talk or GSM CW signals (2 W/kg) / sham exposures, together with positive controls exposed to 4 Gy x-rays and unirradiated controls. Data show as total values ± SE for three experiments of FITC$^+$ cells. p values for each pair of exposed/sham data points are also shown above the exposed data point.
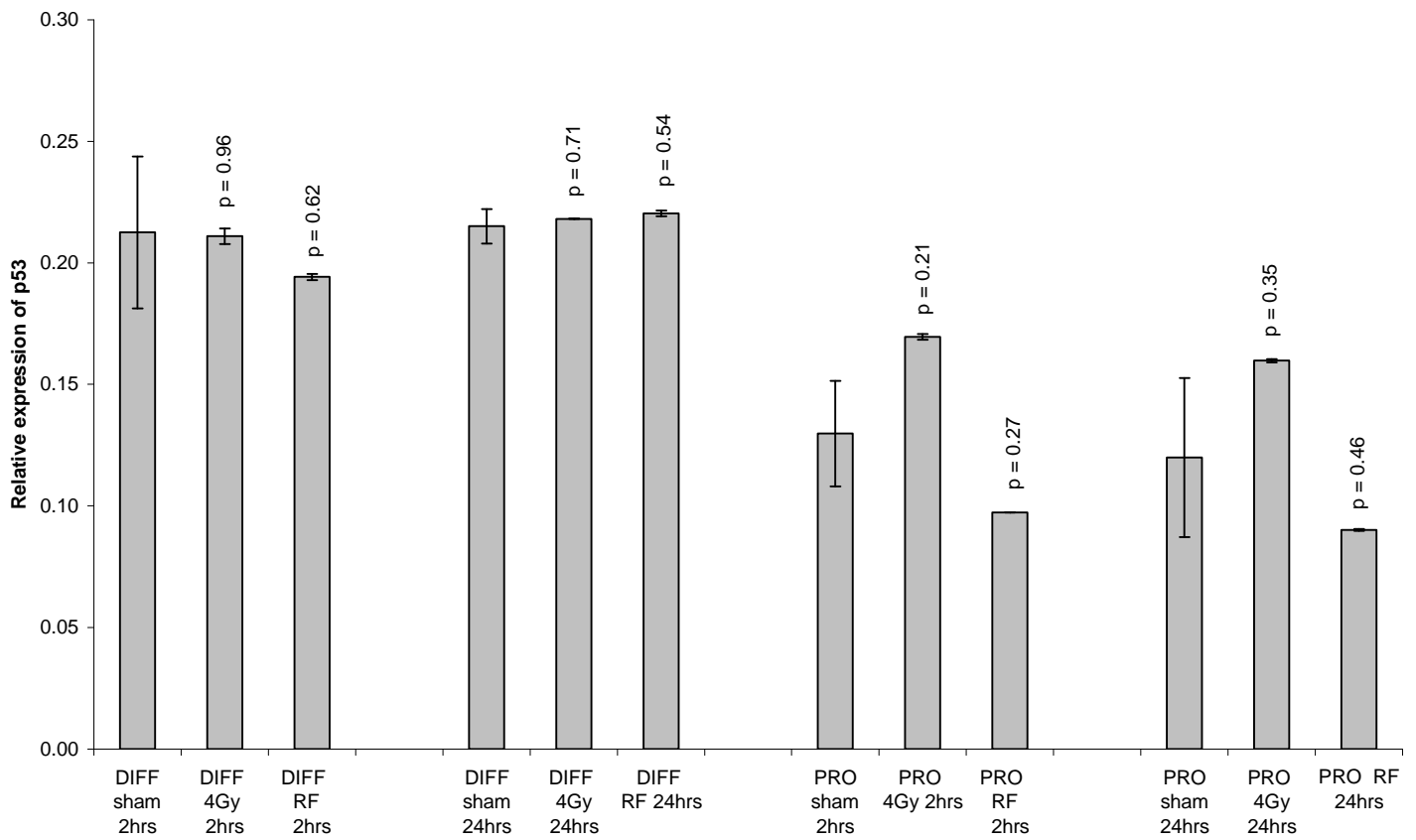
Figure 7. Measurement of the relative gene expression of *p53* in differentiated and proliferating N2a cells following a 24 hour exposure to a 935MHz GSM basic signal with a SAR of 2 W/kg at 2 and 24 hours post exposure. p values for the exposed data points compared to the relevant sham are also shown.
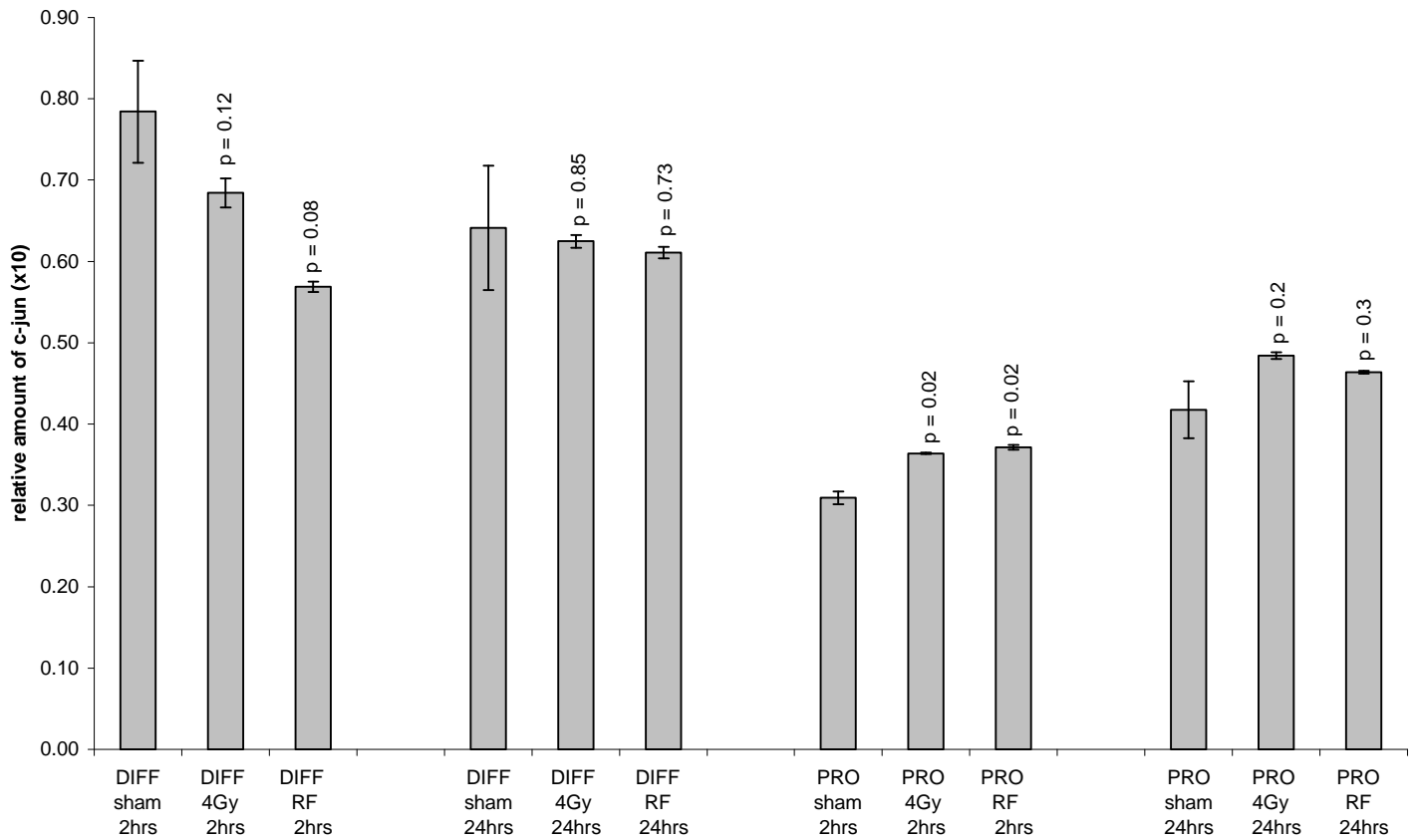
Figure 8. Measurement of the relative gene expression of *c-jun* in differentiated and proliferating N2a cells following a 24 hour exposure to a 935MHz GSM basic signal with a SAR of 2 W/kg at 2 and 24 hours post exposure. p values for exposed data points compared to the relevant sham are also shown.
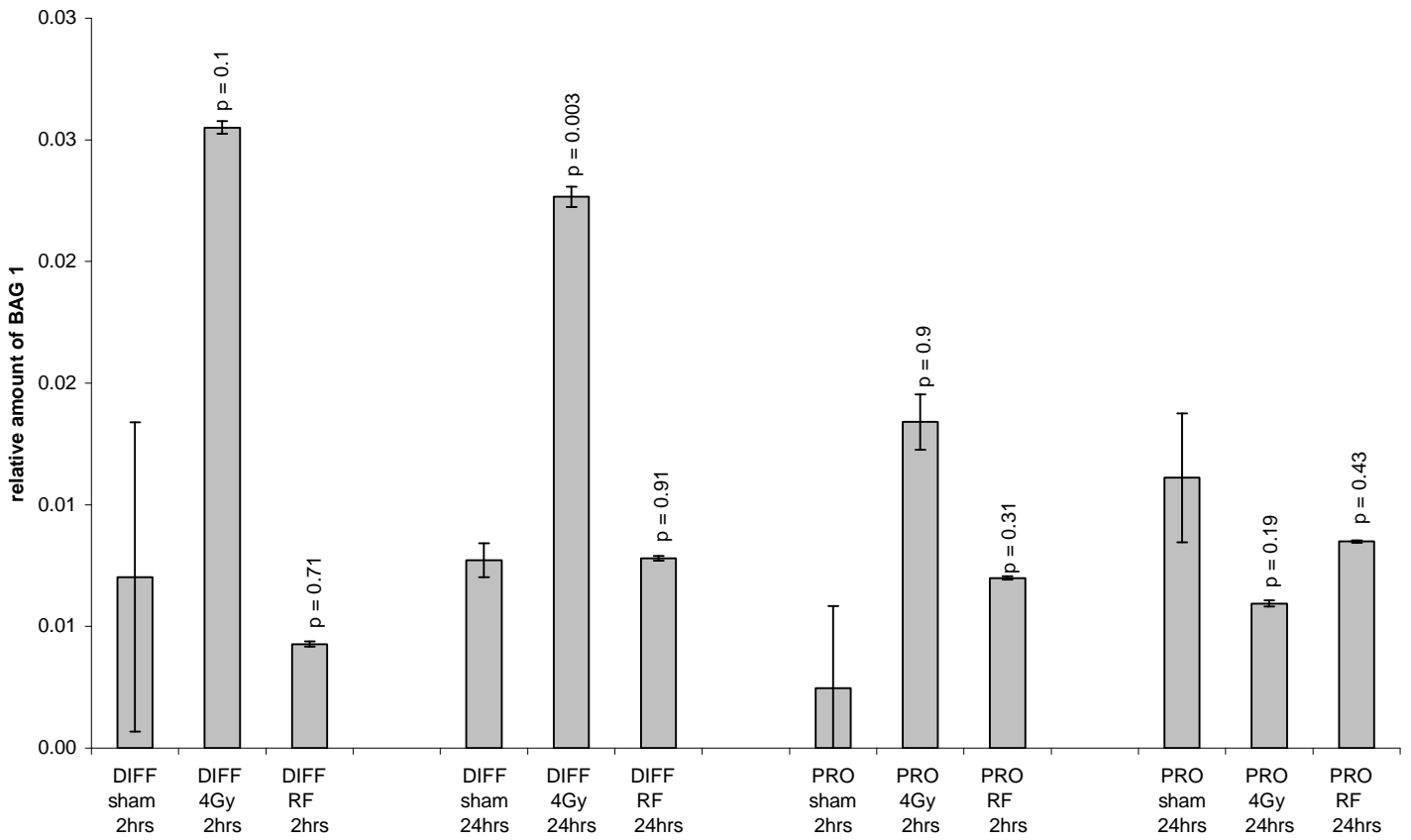
Figure 9. Measurement of the relative gene expression of *bag-1* in differentiated and proliferating N2a cells following a 24 hour exposure to a 935MHz GSM basic signal with a SAR of 2 W/kg at 2 and 24 hours post exposure. p values for exposed data points compared to the relevant sham are also shown.