Comparison of S-nitrosoglutathione- and staurosporine-induced apoptosis in human neural cells

Sodja, Caroline; Ribecco-Lutkiewicz, Maria; Haukenfrers, Julie; Merchant, Fahar; Costain, Willard J.; Bani-Yaghoub, Mahmud

Publisher's version / Version de l'éditeur:
https://doi.org/10.1139/cjpp-2014-0053
Canadian Journal of Physiology and Pharmacology, 92, 12, pp. 1001-1011, 2014-12

Canadian Journal of Physiology and Pharmacology

Canadian Journal of
Physiology and Pharmacology
Revue canadienne de
physiologie et pharmacologie

Characterization of S-nitrosoglutathione- and staurosporine-induced apoptosis in human neural cells

Journal:	*Canadian Journal of Physiology and Pharmacology*
Manuscript ID:	Draft
Manuscript Type:	Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Sodja, Caroline; National Research Council, Human Health Therapeutics Ribecco-Lutkiewicz, Maria; National Research Council, Human Health Therapeutics Costain, Willard; National Research Council, Human Health Therapeutics Bani, Mahmud; National Research Council, Human Health Therapeutics; University of Ottawa, Cellular and Molecular Medicine
Keyword:	caspase-3, apoptosis, S-nitrosoglutathione, staurosporine, neural cells

Characterization of *S*-nitrosoglutathione- and staurosporine-induced apoptosis in human neural cells

Caroline Sodja, Maria Ribecco-Lutkiewicz, Will J. Costain*, Mahmud Bani-Yaghoub[1,2,*]

[1]Department of Translational Biosciences,

Human Health Therapeutics,

National Research Council Canada,

1200 Montreal Road, Ottawa, Ontario, Canada, K1A0R6

[2]Department of Cellular and Molecular Medicine,

University of Ottawa, Ottawa,

Ontario, Canada, K1H 8M5

*Corresponding authors

E-mail: mahmud.bani@nrc.ca

will.costain@nrc.ca

ABSTRACT:

A number of agents targeting cell death have been used extensively to establish cell-based assays for testing new drugs designed to modulate various cellular functions in the nervous system. In particular, *S*-nitrosoglutathione (GSNO) is known for endogenously producing anti-oxidative compounds that control nitric oxide signaling as well as the function of a variety of proteins through *S*-nitrosylation. Currently, a number of rodent cell lines are used to study the effects of GSNO on apoptotic pathways. However, the mechanisms of action by which GSNO induces cell death remains to be evaluated in human cells and in parallel with other agents commonly used to study apoptosis.

In this study, we aimed to compare the pro-apoptotic effects of GSNO and staurosporine (STS) on human neural progenitors (NT2, hNP1) and neuroblasts (SH-SY5Y). We show that neural progenitors and neuroblasts exhibit comparable levels of susceptibility to GSNO- and STS-induced cell death. In particular, apoptosis was observed following treatment with either GSNO or STS, as demonstrated by condensed nuclei and caspase-3 activation. Examination of BAX and BCLXL levels revealed a differential pattern of activation between NT2 and hNP1 neural progenitors and SH-SY5Y neuroblasts, suggesting a potential mechanistic difference in apoptotic responses related to development stage. Assessment of ERK and p-ERK levels after treatment with GSNO or STS provided further evidence that these cells exhibit differential responsiveness to apoptotic inducers. These results were complemented by mitochondrial membrane potential analysis, revealing that NT2 and hNP1 cells undergo mitochondrial hyperpolarization in response to short-term exposure to STS prior to undergoing subsequent depolarization. However, hyperpolarization was not observed in neuroblasts (SH-SY5Y).

This is the first study to report differences in the apoptotic responses to GSNO and STS in three complementary human neural cell lines. Furthermore, these cells can be used as useful tools in

cell pharmacological paradigms in which susceptibility to apoptotic-inducing agents needs to be assessed at different stages of neural cell fate commitment and differentiation.

KEY WORDS:

caspase-3, apoptosis, *S*-nitrosoglutathione, staurosporine, NT2, hNP1, SH-SY5Y

INTRODUCTION:

Apoptosis or programmed cell death is a complex process that is known for its essential roles in regulating cellular proliferation, tissue development and senescence in multicellular organisms. While occurring routinely in all healthy tissues to maintain homeostasis, deregulation of apoptosis is considered as a common feature in pathological conditions, including neurodegenerative diseases. The apoptotic process can be initiated by a wide range of factors such as hypoxia, UV irradiation, hyperosmolality and various chemical agents. Many of these factors are known to mediate cell death in the nervous system, using well-established extrinsic and intrinsic pathways. In particular, there is compelling evidence that extracellular and intracellular redox environments serve as important regulators of apoptosis in the brain (Diers et al. 2013; Federico et al. 2012). Indeed, the generation of reactive oxygen species is a well-known component of certain apoptotic pathways and depletion of glutathione (GSH) is commonly observed as an early event following exposure to apoptotic stimuli (Dukhande et al. 2013).

The apoptotic process itself is characterized by a series of morphological and biochemical features including changes in mitochondrial membrane potential, mitochondrial transition pore opening, phosphatidylserine translocation to outer cell membrane, caspase activity, cytoskeleton disruption and breakdown, metabolic activity, chromatin condensation, DNA fragmentation, endonuclease activation, cell shrinkage and ultimately cell fragmentation. From a molecular mechanism standpoint, caspases 3, 6, and 7 are known to play a central role in the apoptotic cascade, as their activation is a convergence point for intrinsic and extrinsic initiators and represents the beginning of the execution phase (Franco and Cidlowski 2009). Once activated, caspase 3 (CASP3) cleaves a plethora of proteins that containing its recognition motifs (Fang et al. 2006; Han et al. 2013; Mahrus et al. 2008). Recent proteomic studies have led to the revision of what is accepted to be the canonical CASP3 recognition motif (DEVD), with the identification of a greater diversity of recognition sites (Mahrus et al. 2008) and cell type specificity of motif prevalence (Han et al. 2013). The activation of CASP3 has been known to be at least partially

mediated by the BCL2 family of proteins, which includes both pro- and anti-apoptotic members (Chan and Yu 2004; Snigdha et al. 2012). Disruption of the balance of pro- and anti-apoptotic protein levels tilts the system toward either apoptosis or survival. In particular, the BAX/BCLXL ratio is considered as an important regulatory point for the apoptotic cascade (Renault et al. 2013). While the level of CASP3 and the ratio of BAX/BCLXL are viewed as defining parameters of apoptosis, emerging evidence supports a complementary role for caspase-independent apoptotic mechanisms. For instance, activation of extracellular signal-regulated kinase 1-2 (ERK1-2), a molecule known for mediating growth factor stimulated proliferation and differentiation, can cause cell death in the absence of CASP3 activation (Cheung and Slack 2004; Subramaniam and Unsicker 2010). Similarly, some mitochondrial factors are also known to be capable of inducing apoptosis and DNA fragmentation, independent of CASP3 activation (Cheung and Slack 2004; Subramaniam and Unsicker 2010).

In recent years, significant progress has been made towards our understanding of the regulatory mechanisms by which cytotoxic factors mediate apoptosis in the brain. However, only a few studies have specifically focused on the effects of *S*-nitrosoglutathione (GSNO) on neural cell death. GSNO is produced by the interaction of NO with reduced GSH (Broniowska et al. 2013) and it is known to induce *S*-nitrosylation of both cell surface and intracellular proteins without undergoing direct cellular internalization (Broniowska, Diers, and Hogg 2013; Yin et al. 2013; D.-H. Liu et al. 2013; Qu et al. 2012). Furthermore, several groups have reported anti-inflammatory and neuroprotective functions of GSNO *in vitro* and *in vivo* (Andoh et al. 2000; Khan et al. 2009; Qu et al. 2012; Won et al. 2013; Yin et al. 2013), suggesting that GSNO has the potential to be used clinically in the future. However, the limited findings from other laboratories focusing on GSNO-mediating cell death have challenged this notion, proposing that GSNO may work on a context- and dose-dependent manner (Franco and Cidlowski 2009; He et al. 2004; Q.-B. Liu et al. 2010; Pannu and Singh 2006). Pannu and Singh (2006) have shown that the NO generated from GSNO acts as second messenger, regulating the expression of specific

proteins. Thus, the environment in which NO is produced may determine its beneficial and detrimental effects. Furthermore, there is increasing recognition of the significant role of aberrant *S*-nitrosylation in the development of a variety of neurodegenerative diseases (Nakamura et al. 2013). These multi-faceted aspects of GSNO make its therapeutic potential in neurodegeneration and other diseases complicated without a careful examination of its effects on human cells (Nakamura et al. 2012).

To properly assess the role of GSNO in vitro, cells need to be selected based on their origin, physiological properties and applicability to relevant assays. Added to these considerations should be an understanding of the cell-type specific differences in responses, as well as the mechanisms underlying these differences. In this study, we aimed to compare the responses of three complementary human neural cell types to GSNO and staurosporine (STS), which is a potent non-specific protein kinase inhibitor known to induce apoptosis (Lopez et al. 2013; McGinnis et al. 1999; Tamaoki and Nakano 1990). We were also interested in assessing the degree of similarity in apoptotic responses to GSNO and STS between human neural progenitors (Ntera2/D1 and hNP1) and human neuroblasts (SH-SY5Y cells), which represent different stages of cell differentiation and possibly susceptibility to apoptosis. Our findings not only provide novel information about the suitability of these cells to study apoptotic cell death mechanisms, but also they facilitate developing strategies to design therapeutic approaches targeting pathological conditions in the nervous system.

MATERIALS AND METHODS:

Cell Systems - Two human neural cell lines, human embryonal teratocarcinoma Ntera2/D1 (NT2) cells (Stratagene, La Jolla, CA) and SH-SY5Y neuroblastoma cells (ATTC, Manassas, VA), were maintained in high glucose Dulbecco's Modified Eagles medium (HG-DMEM) (Life Technologies, Burlington, ON) with 10% fetal bovine serum (Wisent, Saint Bruno, QC). Cells

were trypsinized, plated in 12 well plates at a density of 50,000 cells/well and incubated for two days prior to treatments.

Human neural progenitor cells (hNP1, ArunA Biomedical, Athens, GA) were grown in AB2 Basal Neural Medium supplemented with ANS Neural Medium Supplement, 20 ng/ml bFGF, 2 mM L-glutamine, and 10 ng/ml LIF on 6 well plates coated with Matrigel (VWR, Mississauga, ON). hNP1 cells were replenished with supplemented AB2 medium every two days, and were maintained in culture until passage 9. Cells were dislodged by gentle trituration and were plated at a 1:3 dilution in 12 well plates coated with Matrigel.

Drug Treatments - GSNO was prepared fresh for each experiment. Reduced glutathione (GSH) (Sigma-Aldrich, Oakville, ON) was dissolved in 1 M $NaNO_2$ (VWR) to produce a 1 M GSNO stock. NT2, SH-SY5Y, and hNP1 cells were treated for 22 hrs with increasing concentrations of GSNO or Staurosporine (STS) (Sigma) in complete medium with serum to determine which concentrations resulted in approximately 50% cell death. These concentrations were used for all subsequent experiments and are listed in **Table 1**.

Cell Viability Assays - The 5-carboxyfluorescein diacetate (CFDA) assay was used to determine cell viability in 22 hr control, GSNO-, and STS-treated cultures (Sandhu et al. 2005). Cells were incubated with 2.5 μg/mL of CFDA (Molecular Probes) in Earle's Balanced Salt Solution (Sigma) at 37°C for 20 min. The fluorogenic CellEvent CASP3/7 detection assay (Molecular Probes, Eugene, OR) was used to detect CASP3 activation after 22 hr in control, GSNO, and STS treated cultures by adding 2 μL of the reagent to live cells and incubating for 30 minutes. For both assays, cells were imaged using an Axiovert 200M microscope (Zeiss) and fluorescence was quantified using a CytoFluor™ 2300/2350 fluorescence measurement system (Millipore, Billerica, MA) with excitation filter 480 ± 20 and emission filter 530 ± 25. To examine nuclear morphology of control, GSNO and STS treated cells, cultures were fixed for 8 minutes at room temperature with Genofix (DNA Genotek Inc., www.DNAGenotek.com), stained with Hoechst

33258 (Sigma) and mounted in DAKO fluorescent mounting medium. Cells were imaged using an Axiovert 200M microscope (Zeiss). Images were taken with Axiovision 3.0 software (Zeiss, Thornwood, NY) and were processed using Adobe Photoshop (Adobe Systems Incorporated).

Western blotting - Cells were treated with GSNO or STS for 4, 7, or 22h. Cells were lysed in RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% Deoxycholate, 1% triton X-100) and proteins were separated by 12% SDS-PAGE. Proteins were transferred to nitrocellulose and membranes were probed with rabbit polyclonal BAX, rabbit polyclonal BCLXL, rabbit polyclonal cleaved caspase, rabbit polyclonal Phospho-p44/42 MAPK (ERK1/2), rabbit polyclonal p44/42 MAPK (ERK1/2) (all from Cell Signalling Technology, Danvers, MA) or mouse monoclonal β-actin (Sigma), followed by HRP-conjugated secondary antibodies. Membranes were developed with Immunstar ECL kit (Biorad, Mississauga, ON).

Mitochondrial permeability - Cells in 12 well plates were treated with GSNO or STS for 1, 4 or 22h in complete medium, and then loaded with TMRM (200nM from a 2mM stock in DMSO) for 30 min in Earle's Balanced Salt Solution (EBSS) at 37°C in a 5% CO2 incubator. Cells were washed twice with EBSS and TMRM staining was quantitated by fluorescence intensity using a CytoFluorTM 2300/2350 fluorescence measurement system with excitation filter 560 ± 20 and emission filter 620 ± 40.

Statistical analysis - The data were expressed as mean ± S.E.M. and analyzed using a 2-way ANOVA with Dunnett's multiple comparisons post-hoc test to assess treatment effects.

RESULTS:

Comparative analysis of cell death in response to GSNO and STS

The CFDA assay was used to determine the effect of GSNO and STS on cell viability in NT2 and hNP1 neural progenitors and SH-SY5Y neuroblasts (**Figure 1**). CFDA is a non-fluorescent hydrophobic dye that is cleaved inside live cells forming a fluorescent hydrophillic anion, 5(6)-

carboxyfluorescein. This compound remains inside the cytoplasm of live cells and exhibits fluorescence (Petroski and Geller 1994). Representative CFDA staining is illustrated in **Figure 1** for 22 hr control, GSNO and STS treated cells. The CFDA images were merged with the corresponding phase contrast images to show the entire cell population. Labelling was consistent for the three cell types examined, and for both types of treatments. In both control and GSNO- or STS-treated cultures, cells with a normal morphology were labelled, whereas condensed rounded dead cells lacked fluorescence (**Fig. 1A, 1D, 1G**). CFDA fluorescence was quantified in the three cell types treated with increasing doses of GSNO or STS. All three cell types demonstrated a dose-dependent reduction in cell viability in response to the treatments. However, there were differential sensitivities among the cell types. Comparative analysis of concentrations required to reduce cell viability by 50% showed that SH-SY5Y cells were the most sensitive to GSNO (0.25 mM for approx. 50% death) (**Fig. 1E**). Whereas, NT2 and hNP1 cells required double (0.5 mM) and quadruple (1 mM) this concentration for a similar reduction in viability, respectively (**Fig. 1B, 1H**). In contrast, NT2 cells were the most sensitive to STS treatment (10 nM for approx. 50% death) (**Fig. 1C**). SH-SY5Y and hNP1 cells were somewhat similar in their responses to STS (50 nM and 35 nM, respectively, for 50% cell death) (**Fig. 1F, 1I**). These results allowed for a careful selection of GSNO and STS concentrations for subsequent experiments, as summarized in **Table 1**.

GSNO and STS induce apoptosis in neural progenitors and neuroblasts

The ability of STS to induce apoptosis in numerous cell lines is well known. However, it is unclear if GSNO-induced cell death is mediated through apoptosis in neural progenitors and neuroblasts. To address this issue, we performed Hoechst 33258 staining to examine the morphology of chromatin after treatment of cells with GSNO or STS. Nuclei with classical apoptotic morphology were observed in all three cell types following 22 hr treatment with either GSNO or STS (**Fig. 2A, 2D, and 2G**). To further confirm that apoptosis is the mode of cell death occurring, we examined the induction of active CASP3 under the same conditions. The

CellEvent assay was used to identify individual cells with activated CASP3. The assay reagent contains a peptide with the cleavage site for caspase3/7 (DEVD) conjugated to a nucleic acid binding dye. While conjugated, the dye is non-fluorescent. Following cleavage, the dye is able to bind to DNA, producing green fluorescence. Representative CellEvent images are shown in **Figure 2B, 2E and 2H** for 22 hr control, GSNO- and STS-treated cells. The CellEvent images were merged with their corresponding phase contrast images to show the entire cell population. Both GSNO- and STS-treated cultures demonstrated cell populations exhibiting green fluorescence, indicative of the presence of active CASP3. Moreover, it was the rounded collapsed cells that fluoresced with the CellEvent assay, confirming that these cells were apoptotic (**Fig. 2A-B, 2D-E, 2G-H**). We determined that both GSNO and STS significantly elevated the levels of active CASP3 in all the three cell types, (1.5 fold for SH-SY5Y and 2 fold for NT2 and hNP1, **Figure 2C, 2F, 2I**). These results confirmed that the GSNO and STS concentrations chosen to induce 50% cell death produced comparable levels of CASP3 activation in all three cell types.

GSNO and STS exhibit differential effects on apoptotic mediators

We aimed to identify potential mechanistic differences related to the involvement of BCL2 proteins in GSNO- and STS-induced apoptosis. We examined the effect of GSNO and STS on the levels of the pro-apoptotic protein BAX and the anti-apoptotic protein BCLXL. Due to the complex nature of the interactions among BCL2 proteins in controlling apoptosis, it has been demonstrated that the absolute levels of individual proteins are less important than the interplay among various family members (Shamas-Din et al. 2013). Therefore we assessed BAX and BCLXL levels in order to determine the BAX/BCLXL ratio, which is more indicative of the state of apoptotic regulation. **Figure 3** shows that 22 hr STS treatment resulted in decreased BAX and BCLXL levels in NT-2 cells (**Fig. 3A**), whereas GSNO treatment did not alter the levels of these proteins. A similar trend was also observed in SH-SY5Y (**Fig. 3B**) and hNP1 (**Fig. 3C**) cells. Interestingly, the BAX/BCLXL ratio was reduced by GSNO and STS treatment in NT2 and

hNP1 cells, but not in SH-SY5Y cells. These results suggest that neural progenitors mount an anti-apoptotic response that is not observed in neuroblast cells.

To better understand the process involved in GSNO- and STS-induced apoptosis, we assessed the levels of these apoptotic markers at time points that preceded the frank apoptosis observed at 22 hrs. The maximal CASP3 activation observed in this study occurred with a 7 hr treatment duration (**Fig. 4A – F**), with no changes observed at 4 hrs (data not shown). Interestingly, GSNO and STS had no discernible effects on BAX and BCLXL levels in all cell types at 7 hrs, with the exception of a reduction in BCLXL levels observed in STS-treated hNP1 cells (**Fig. 4**). This is in stark contrast to the elevated cleaved CASP3 levels observed at 7 hrs. Consistent with the observations at 22 hrs, all cell types responded to GSNO and STS treatment with elevated cleaved CASP3 levels (**Fig. 4D – F**).

Additionally, we examined the levels of ERK and P-ERK to assess the effects of GSNO and STS on MAPK signaling and the potential contribution of caspase-independent apoptotic mechanisms. **Figure 4G – I** shows reduced P-ERK levels in both NT2 and hNP1 cells following GSNO or STS treatment. Notably, GSNO treatment produced a much larger effect on P-ERK, compared with that of STS. Furthermore, ERK level were not appreciably altered by either GSNO or STS treatment. These results suggest that GSNO and STS may differ significantly in the mechanisms employed in producing apoptosis.

Effect of GSNO and STS on mitochondrial polarity

Cell viability is dependent on normal mitochondrial function, as mitochondria mediate oxidative phosphorylation and disruption of the mitochondria results in the release of pro-apoptotic factors. Mitochondrial depolarization is an early event indicative of imminent cell death. Thus, we examined the effects of GSNO and STS on mitochondrial membrane potential using TMRM staining. We examined three time points in an attempt to discern a sequence of events. **Figure 5** shows that significant mitochondrial depolarization was observed at the 22 hr time point in all

cell lines with both GSNO and STS. The magnitude of the depolarization is consistent with the observed degree of cell death (**Fig. 1**) produced by these treatments. Examination of time points prior to peak CASP3 activation at 7 hrs revealed the absence of mitochondrial depolarization. Interestingly, significant STS-induced hyperpolarization was observed in hNP1 cells at 1 and 4 hrs, with a similar tendency observed in NT2 cells, suggesting cell-type specific responses. In comparison, SH-SY5Y cells did not exhibit hyperpolarization. Furthermore, no evidence for GSNO induced hyperpolarization was observed in the three cell types.

DISCUSSION:

The objective of this study was to characterise the susceptibility of human neural cells to GSNO- and STS-induced apoptotic cell death. The project was undertaken for two reasons. First, very little information is available regarding GSNO toxicity in neuroblasts and neural progenitor cells. Secondly, the lack of information on the responses of these cells toward *S*-nitrosylating agents represents a considerable gap in our understanding of the potential applications. An understanding of these responses is particularly important considering the fact that GSNO is currently employed in human clinical trials (Broniowska et al. 2013). While the majority of these trials focus on the effects of GSNO on platelets and the regulation of vascular parameters, off target effects need to be considered as well. Hence, an understanding of potential adverse effects with respect to progenitor cells is vitally important. Furthermore, this study aims to inform the application of human neural cells in pharmacological paradigms with respect to cell-specific signaling and responses to apoptotic stimuli.

GSNO is an endogenous *S*-nitrosothiol (SNO) that is an important component of the nitric oxide signalling pathway. NO is generated by the nitric oxide synthase (NOS) enzymes and exists in cells as a pool of SNOs and NO. SNOs act as endogenous NO carriers and donors, and GSNO in particular serves as a stable and mobile pool of NO capable of transducing NO signalling. Thus GSNO, and other NO donors, constitute a redox-responsive signalling system capable of

modulating the activity of a plethora of proteins through *S*-nitrosylation of cysteine residues critical to their function.

Here, we demonstrated that the cytotoxic effects of GSNO and STS on neural cells were observed at millimolar and nanomolar concentrations, respectively. While a minor degree of variability in the EC_{50} values was observed, no dramatic differences in susceptibility to either GSNO or STS was observed among the cell types examined (**Table 1**, **Figure 1**). Furthermore, the hallmarks of apoptosis, condensed nuclei and CASP3 activation, were observed in all cell types (**Figure 1 & 2**). These observations indicate that CASP3 activation is a central event in the apoptotic cascades induced by both STS and GSNO. Furthermore, this data is consistent with previous reports of the effect of STS on a variety of cell types (Posadas et al. 2007; Yuste et al. 2002), and represents new information with respect to the effects of GSNO on neural cells.

The BCL2 family of proteins play a prominent role in the regulation of intrinsic apoptosis. We therefore assessed the effects of GSNO and STS on the status of two key members of the BCL2 family. We examined BAX and BCLXL levels using western blot during a period where activated caspase is observed (22 hr). We found that NT2 and hNP1 cells responded similarly with an apparent anti-apoptotic BCL2 response (decreased BAX/BCLXL ratio). Conversely, no evidence for an anti-apoptotic response was observed in SH-SY5Y cells (**Figure 3**). However, in comparison to the time point where the greatest levels of activated CASP3 was observed, 7 hrs, no anti-apoptotic alterations in BCL2 proteins were observed (**Figure 4**). If anything, the reduction in BCLXL levels observed at 7 hrs would contribute to the apoptotic signalling. Given that CASP3 activation occurs long before the presumed anti-apoptotic BCL2 response observed at 22 hrs, the alterations in BCL2 proteins seems to be insufficient to protect NT2 and hNP1 cells from ongoing apoptotic signalling. Furthermore, these results suggest that cell-type specific differences exist in the molecular mechanisms elicited in human neural progenitor cells versus neuroblasts.

The effect of GSNO and STS on ERK signalling, which is typically involved in proliferation and differentiation, were also examined. We found that GSNO produced a very strong reduction in P-ERK levels in NT2 and hNP1 cells, and a much less pronounced effect on SH-SY5Y cells (**Figure 4**). This observation is consistent with the report by Feng et al (2013), who reported that P-ERK levels are decreased following treatment with the *S*-nitrosylating agent SNP. Furthermore, these authors demonstrated that SNP treatment produced *S*-nitrosylation of ERK, which results in reduced ERK activity. Additionally, STS produced a similar, yet less pronounced, pattern on P-ERK levels. When the effects of GSNO and STS are compared, it appears that STS produces a larger effect on CASP3 activation, while GSNO produces a more pronounced effect on P-ERK levels. This suggests that the GSNO and STS produce discreet effects on cellular signalling and elicit apoptosis in a mechanistically distinct manner.

The role of the mitochondria in mediating apoptotic signals is well established. Therefore, we assessed the effects of GSNO and STS treatment on mitochondrial polarity as a marker of the participation of mitochondrial-derived factors on apoptotic signalling. We found that neither GSNO nor STS induced mitochondrial depolarization prior to or during peak CASP3 activation (**Figure 5**). However, by 22 hours, both GSNO and STS had produced varying degrees of mitochondrial depolarization. This indicates that mitochondrial depolarization was not required to elicit CASP3 activation and apoptosis. This is consistent with previous reports that indicate that mitochondrial depolarization is not required for, nor necessarily precedes apoptosis (Budd et al. 2000; Krohn et al. 1999). Indeed, Budd et al (2000) reported that mitochondrial depolarization was not necessary for STS-induced cytochrome C release, which was dependent on caspase 8 and Bid activation. Furthermore, we observed that STS treatment produced a significant hyperpolarization of hNP1 cells at 1 and 4 hrs. Mitochondrial hyperpolarization is a known indicator of mitochondrial reactive oxygen species (ROS) generation (Sanderson et al. 2013) and STS-induced ROS generation has been previously reported (Ribeiro et al. 2013). This supports a role for ROS in mediating STS activation of CASP3, which is consistent with reports of CASP3

and CASP9 activation upon H_2O_2 treatment (Nomura et al. 2013). Furthermore, the lack of evidence for GSNO-induced ROS production is consistent the report of Diers et al. (2013), who reported that treatment with the nitrosylating agent CysNO impaired mitochondrial respiration but did not induce ROS production. Therefore, it appears that ROS generation contributes to the early stages of STS-induced cellular injury in neural progenitor cells, but not neuroblasts. Furthermore, GSNO-induced apoptotic cell death does not appear to involve ROS generation.

The observations presented here led us to develop a model of the potential mechanism for GSNO-induced apoptosis (**Figure 6A-B**). Under normal conditions, the levels of active CASP3 are maintained at sub-apoptotic levels, in part, by the action of inhibitors of apoptosis (IAP) proteins, such as the X-linked inhibitor of apoptosis (XIAP) (**Figure 6A**). XIAP acts as an E3 ubiquitin ligase that efficiently targets active CASP3 for proteasome degradation, thereby preventing the accumulation of active CASP3 (Nakamura et al. 2012). *S*-Nitrosylaiton of XIAP (SNO-XIAP) results in inhibition of its E3 ligase activity and consequently inhibition of active CASP3 degradation (Nakamura and Lipton 2013). Interestingly, the *S*-nitrosylated form of active CASP3 (SNO-aCASP3) and is capable of transnitrosylating XIAP (Nakamura et al. 2010), which effectively enables the accumulation of active CASP3. Thus, the observed GSNO-induced activation of CASP3 is likely due to the inhibition of active CASP3 degradation (**Figure 6B**).

Cell-based assays of are commonly used to evaluate a wide variety of pharmacological agents, particularly in the field of neurodegenerative diseases. The choice of cell line is typically based upon the relevance to the disease being investigated; however, such choices may also be based on the utility of a cell line in specific assays. While practical considerations will always be part of the decision process, knowledge of cell-type specific responses needs to be employed to ensure that the model is appropriate from a mechanistic standpoint. The results of our study are an illustration of this concept. We found that there were clear mechanistic differences between neural progenitors and neuroblasts in their responses to STS and GSNO (**Figure 6C**). STS induced mitochondrial hyperpolarization in neural progenitor cells, but not in neuroblasts,

indicating that the generation of ROS is a component of STS-induced apoptosis in progenitor cells. Similarly, we found that the effect of GSNO treatment on ERK signaling was far more pronounced in neural progenitors than in neuroblasts (**Figure 6C**). Again, this notion indicates that GSNO-induced toxicity also displayed cell-type specificity. In the present examples, agents that directly affect ROS or ERK signaling would be expected to produce differential effects in neuroblasts when compared to neural progenitors.

Previous studies have demonstrated that the •NO donor GSNO is not cytotoxic or pro-apoptotic at 30 μM, and can counteract serum deprivation induction of •OH damage (Andoh et al. 2000). Here, we have demonstrated that GSNO can induce apoptosis in human neural cells in the low mM range. Furthermore, we have also shown that the apoptotic signalling mediated by GSNO is distinct from that of STS and that the apoptotic signalling pathways are not dependent on mitochondrial depolarization. Additionally, we have presented evidence that the susceptibility of human neural cells to GSNO and STS is likely to be affected by their state of differentiation. Taken together, this report informs the suitability of these *in vitro* models as tools in cell pharmacological paradigms focusing on apoptosis.

ACKNOWLEDGEMENTS:

The authors would like to thank Dr. Jagdeep Sandhu for useful suggestions regarding apoptosis assays.

REFERENCES:

Andoh, T., Lee, S. Y., and Chiueh, C. C. (2000). Preconditioning regulation of bcl-2 and p66shc by human NOS1 enhances tolerance to oxidative stress. *FASEB Journal□: Official Publication of the Federation of American Societies for Experimental Biology*, *14*(14), 2144–6. doi:10.1096/fj.00-0151fje

Broniowska, K. A., Diers, A. R., and Hogg, N. (2013). S-nitrosoglutathione. *Biochimica et Biophysica Acta*, *1830*(5), 3173–81. doi:10.1016/j.bbagen.2013.02.004

Budd, S. L., Tenneti, L., Lishnak, T., and Lipton, S. A. (2000). Mitochondrial and extramitochondrial apoptotic signaling pathways in cerebrocortical neurons. *Proceedings of the National Academy of Sciences of the United States of America*, *97*(11), 6161–6. doi:10.1073/pnas.100121097

Chan, S.-L., and Yu, V. C. (2004). PROTEINS OF THE BCL-2 FAMILY IN APOPTOSIS SIGNALLING: FROM MECHANISTIC INSIGHTS TO THERAPEUTIC OPPORTUNITIES. *Clinical and Experimental Pharmacology and Physiology*, *31*(3), 119–128. doi:10.1111/j.1440-1681.2004.03975.x

Cheung, E. C. C., and Slack, R. S. (2004). Emerging role for ERK as a key regulator of neuronal apoptosis. *Science's STKE□: Signal Transduction Knowledge Environment*, *2004*(251), PE45. doi:10.1126/stke.2512004pe45

Diers, A. R., Broniowska, K. A., and Hogg, N. (2013). Nitrosative stress and redox-cycling agents synergize to cause mitochondrial dysfunction and cell death in endothelial cells. *Redox Biology*, *1*(1), 1–7. doi:10.1016/j.redox.2012.11.003

Dukhande, V. V, Kawikova, I., Bothwell, A. L. M., and Lai, J. C. K. (2013). Neuroprotection against neuroblastoma cell death induced by depletion of mitochondrial glutathione. *Apoptosis□: An International Journal on Programmed Cell Death*, *18*(6), 702–12. doi:10.1007/s10495-013-0836-4

Fang, B., Boross, P. I., Tozser, J., and Weber, I. T. (2006). Structural and Kinetic Analysis of Caspase-3 Reveals Role for S5 Binding Site in Substrate Recognition. *Journal of Molecular Biology*, *360*(3), 654–666. Retrieved from http://www.sciencedirect.com/science/article/pii/S002228360600622X

Federico, A., Cardaioli, E., Da Pozzo, P., Formichi, P., Gallus, G. N., and Radi, E. (2012). Mitochondria, oxidative stress and neurodegeneration. *Journal of the Neurological Sciences*, *322*(1), 254–262. Retrieved from http://www.sciencedirect.com/science/article/pii/S0022510X12002584

Feng, X., Sun, T., Bei, Y., Ding, S., Zheng, W., Lu, Y., and Shen, P. (2013). S-nitrosylation of ERK inhibits ERK phosphorylation and induces apoptosis. *Scientific Reports*, *3*, 1814. doi:10.1038/srep01814

Franco, R., and Cidlowski, J. A. (2009). Apoptosis and glutathione: beyond an antioxidant. *Cell Death and Differentiation*, *16*(10), 1303–14. doi:10.1038/cdd.2009.107

Han, M.-H., Jiao, S., Jia, J.-M., Chen, Y., Chen, C. Y., Gucek, M., Markey, S. P., and Li, Z. (2013). The Novel Caspase-3 Substrate Gap43 is Involved in AMPA Receptor Endocytosis and Long-Term Depression. *Molecular & Cellular Proteomics□: MCP*, *12*(12), 3719–31. doi:10.1074/mcp.M113.030676

He, J., Kang, H., Yan, F., and Chen, C. (2004). The endoplasmic reticulum-related events in S-nitrosoglutathione-induced neurotoxicity in cerebellar granule cells. *Brain Research*, *1015*(1-2), 25–33. doi:10.1016/j.brainres.2004.04.022

Khan, M., Im, Y.-B., Shunmugavel, A., Gilg, A. G., Dhindsa, R. K., Singh, A. K., and Singh, I. (2009). Administration of S-nitrosoglutathione after traumatic brain injury protects the neurovascular unit and reduces secondary injury in a rat model of controlled cortical impact. *Journal of Neuroinflammation*, *6*, 32. doi:10.1186/1742-2094-6-32

Krohn, A. J., Wahlbrink, T., and Prehn, J. H. M. (1999). Mitochondrial Depolarization Is Not Required for Neuronal Apoptosis. *J. Neurosci.*, *19*(17), 7394–7404. Retrieved from http://www.jneurosci.org/content/19/17/7394.long

Liu, D.-H., Yuan, F.-G., Hu, S.-Q., Diao, F., Wu, Y.-P., Zong, Y.-Y., Song, T., Li, C., and Zhang, G.-Y. (2013). Endogenous nitric oxide induces activation of apoptosis signal-regulating kinase 1 via S-nitrosylation in rat hippocampus during cerebral ischemia-reperfusion. *Neuroscience*, *229*, 36–48. doi:10.1016/j.neuroscience.2012.10.055

Liu, Q.-B., Liu, L.-L., Lu, Y.-M., Tao, R.-R., Huang, J.-Y., Shioda, N., Moriguchi, S., Fukunaga, K., Han, F., and Lou, Y.-J. (2010). The induction of reactive oxygen species and loss of mitochondrial Omi/HtrA2 is associated with S-nitrosoglutathione-induced apoptosis in human endothelial cells. *Toxicology and Applied Pharmacology*, *244*(3), 374–84. doi:10.1016/j.taap.2010.02.004

Lopez, M. S., Choy, J. W., Peters, U., Sos, M. L., Morgan, D. O., and Shokat, K. M. (2013). Staurosporine-derived inhibitors broaden the scope of analog-sensitive kinase technology. *Journal of the American Chemical Society*, *135*(48), 18153–9. doi:10.1021/ja408704u

Mahrus, S., Trinidad, J. C., Barkan, D. T., Sali, A., Burlingame, A. L., and Wells, J. A. (2008). Global Sequencing of Proteolytic Cleavage Sites in Apoptosis by Specific Labeling of Protein N Termini. *Cell*, *134*(5), 866–876. Retrieved from http://www.sciencedirect.com/science/article/pii/S0092867408010209

McGinnis, K. M., Gnegy, M. E., and Wang, K. K. (1999). Endogenous bax translocation in SH-SY5Y human neuroblastoma cells and cerebellar granule neurons undergoing apoptosis. *Journal of Neurochemistry*, *72*, 1899–1906.

Nakamura, T., Cho, D.-H., and Lipton, S. A. (2012). Redox regulation of protein misfolding, mitochondrial dysfunction, synaptic damage, and cell death in neurodegenerative diseases. *Experimental Neurology*, *238*(1), 12–21. Retrieved from http://www.sciencedirect.com/science/article/pii/S0014488612002749

Nakamura, T., and Lipton, S. A. (2013). Emerging role of protein-protein transnitrosylation in cell signaling pathways. *Antioxidants & Redox Signaling*, *18*(3), 239–49. doi:10.1089/ars.2012.4703

Nakamura, T., Tu, S., Akhtar, M. W., Sunico, C. R., Okamoto, S., and Lipton, S. A. (2013). Aberrant Protein S-Nitrosylation in Neurodegenerative Diseases. *Neuron*, *78*(4), 596–614. Retrieved from http://www.sciencedirect.com/science/article/pii/S089662731300398X

Nakamura, T., Wang, L., Wong, C. C. L., Scott, F. L., Eckelman, B. P., Han, X., Tzitzilonis, C., Meng, F., Gu, Z., Holland, E. A., Clemente, A. T., Okamoto, S., Salvesen, G. S., Riek, R., Yates, J. R., and Lipton, S. A. (2010). Transnitrosylation of XIAP regulates caspase-dependent neuronal cell death. *Molecular Cell*, *39*(2), 184–95. doi:10.1016/j.molcel.2010.07.002

Nomura, K., Lee, M., Banks, C., Lee, G., and Morris, B. J. (2013). An ASK1-p38 signalling pathway mediates hydrogen peroxide-induced toxicity in NG108-15 neuronal cells. *Neuroscience Letters*, *549*, 163–7. doi:10.1016/j.neulet.2013.05.045

Pannu, R., and Singh, I. (2006). Pharmacological strategies for the regulation of inducible nitric oxide synthase: neurodegenerative versus neuroprotective mechanisms. *Neurochemistry International*, *49*(2), 170–82. doi:10.1016/j.neuint.2006.04.010

Petroski, R. E., and Geller, H. M. (1994). Selective labeling of embryonic neurons cultured on astrocyte monolayers with 5(6)-carboxyfluorescein diacetate (CFDA). *Journal of Neuroscience Methods*, *52*(1), 23–32. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8090014

Posadas, I., Vellecco, V., Santos, P., Prieto-Lloret, J., and Ceña, V. (2007). Acetaminophen potentiates staurosporine-induced death in a human neuroblastoma cell line. *British Journal of Pharmacology*, *150*, 577–585. doi:10.1038/sj.bjp.0706993

Qu, Z.-W., Miao, W.-Y., Hu, S.-Q., Li, C., Zhuo, X.-L., Zong, Y.-Y., Wu, Y.-P., and Zhang, G.-Y. (2012). N-methyl-D-aspartate receptor-dependent denitrosylation of neuronal nitric oxide synthase increase the enzyme activity. *PloS One*, *7*(12), e52788. doi:10.1371/journal.pone.0052788

Renault, T. T., Teijido, O., Antonsson, B., Dejean, L. M., and Manon, S. (2013). Regulation of Bax mitochondrial localization by Bcl-2 and Bcl-xL: Keep your friends close but your enemies closer. *The International Journal of Biochemistry & Cell Biology*, *45*(1), 64–67. Retrieved from http://www.sciencedirect.com/science/article/pii/S1357272512003330

Ribeiro, M., Silva, A. C., Rodrigues, J., Naia, L., and Rego, A. C. (2013). Oxidizing effects of exogenous stressors in Huntington's disease knock-in striatal cells--protective effect of

cystamine and creatine. *Toxicological Sciences□: An Official Journal of the Society of Toxicology*, *136*(2), 487–99. doi:10.1093/toxsci/kft199

Sanderson, T. H., Reynolds, C. A., Kumar, R., Przyklenk, K., and Hüttemann, M. (2013). Molecular mechanisms of ischemia-reperfusion injury in brain: pivotal role of the mitochondrial membrane potential in reactive oxygen species generation. *Molecular Neurobiology*, *47*(1), 9–23. doi:10.1007/s12035-012-8344-z

Sandhu, J. K., Sodja, C., Mcrae, K., Li, Y., Rippstein, P., Wei, Y., Lach, B., Lee, F., Bucurescu, S., Harper, M., and Sikorska, M. (2005). Effects of nitric oxide donors on cybrids harbouring the mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS) A3243G mitochondrial DNA mutation. Retrieved from http://www.biochemj.org/bj/391/0191/bj3910191.htm

Shamas-Din, A., Kale, J., Leber, B., and Andrews, D. W. (2013). Mechanisms of action of Bcl-2 family proteins. *Cold Spring Harbor Perspectives in Biology*, *5*(4), a008714. doi:10.1101/cshperspect.a008714

Snigdha, S., Smith, E. D., Prieto, G. A., and Cotman, C. W. (2012). Caspase-3 activation as a bifurcation point between plasticity and cell death. *Neuroscience Bulletin*, *28*(1), 14–24. doi:10.1007/s12264-012-1057-5

Subramaniam, S., and Unsicker, K. (2010). ERK and cell death: ERK1/2 in neuronal death. *The FEBS Journal*, *277*(1), 22–9. doi:10.1111/j.1742-4658.2009.07367.x

Tamaoki, T., and Nakano, H. (1990). Potent and Specific Inhibitors of Protein Kinase C of Microbial Origin. *Bio/Technology*, *8*(8), 732–735. doi:10.1038/nbt0890-732

Won, J.-S., Kim, J., Annamalai, B., Shunmugavel, A., Singh, I., and Singh, A. K. (2013). Protective role of S-nitrosoglutathione (GSNO) against cognitive impairment in rat model of chronic cerebral hypoperfusion. *Journal of Alzheimer's Disease□: JAD*, *34*(3), 621–35. doi:10.3233/JAD-121786

Yin, X.-H., Yan, J.-Z., Hou, X.-Y., Wu, S.-L., and Zhang, G.-Y. (2013). Neuroprotection of S-nitrosoglutathione against ischemic injury by down-regulating Fas S-nitrosylation and downstream signaling. *Neuroscience*, *248C*, 290–298. doi:10.1016/j.neuroscience.2013.06.012

Yuste, V. J., Sánchez-López, I., Solé, C., Encinas, M., Bayascas, J. R., Boix, J., and Comella, J. X. (2002). The prevention of the staurosporine-induced apoptosis by Bcl-X(L), but not by Bcl-2 or caspase inhibitors, allows the extensive differentiation of human neuroblastoma cells. *Journal of Neurochemistry*, *80*, 126–139.

	Cell Death (EC_{50})	
Cell Type	**GSNO**	**STS**
NT2	0.5 mM	10 nM
hNP1	1.0 mM	35 nM
SH-SY5Y	0.25 mM	50 nM

Table 1. Concentrations of GSNO and STS found to induce 50% cell death in neural progenitor and neuroblast cells after 22 hours of drug treatment. Drug concentrations were derived from data presented in Figure 1, and were used in all subsequent experiments (Figures 2 – 5).

Figure 1. Dose-dependent GSNO- and STS-induced cytotoxicity in human NT2, SH-SY5Y, and hNP1cells. CFDA staining is depicted in panels **A**, **D** and **G** for NT2, SH-SY5Y and hNP1 cells, respectively (scale bar represents 20 μm). Quantitation of CFDA staining performed after 22 hrs of GSNO or STS treatment demonstrates that cell viability decreased in a dose dependent manner with both compounds in all cell types. NT2 cells were differentially susceptible to GSNO (**B**) and STS (**C**), with a slight reduction in cell viability in the presence of STS. In comparison, SH-SY5Y cells were more susceptible to GSNO (**E**) than STS (**F**). In contrast, hNP1 cells were less susceptible to GSNO (**H**) and exhibited STS (**I**) susceptibility comparable to SH-SYS5Y cells.

Figure 2. Comparative analysis of the effects of GSNO- and STS-treatment on apoptosis and CASP3 activation in NT2, SH-SY5Y, and hNP1cells. In all cell types, apoptotic nuclei were clearly visible, using Hoechst 33258 staining, following 22 hr treatment with either GSNO or STS (**Panels A**, **D** and **G**). The microscopic images presented in **Panels B**, **E** and **H** demonstrate alterations in active CASP3 staining resulting from GSNO and STS treatment (scale bar represents 20 μm). CellEvent staining demonstrated that both GSNO and STS significantly increased CASP3 activation in all cell types (**Panels C**, **F** and **I**). The concentrations of GSNO and STS used are as listed in **Table 1**.

Figure 3. Analysis of the effects of GSNO and STS treatment on the expression of apoptosis-regulating proteins in NT2, SH-SY5Y, and hNP1cells. Western blot analysis indicated that BAX and BCLXL levels were reduced following 22 hr STS and to a lesser extent GSNO treatments. Marked reductions in BAX were observed in NT2 (**A**) and hNP1 (**C**) cells, while effects in SH-SY5Y (**B**) cells were minor. BCLXL levels were moderately reduced from control levels in all cell types.

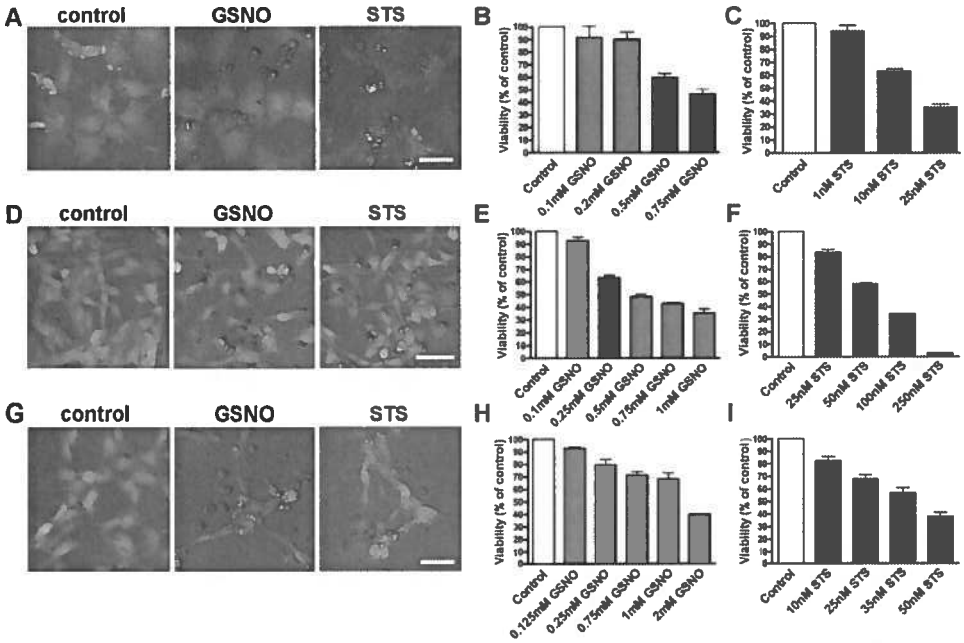
Figure 4. Analysis of the effects of GSNO and STS treatment on the expression of BAX and BCLXL expression in NT2, SH-SY5Y, and hNP1cells. Western blot analysis indicated that BAX

and BCLXL levels were largely unchanged following 7 hr GSNO and STS treatment. In contrast, active CASP3 levels were markedly increased in NT2 (**A**), SH-SY5Y (**B**), and hNP1 (**C**). Quantitative analysis of cleaved CASP3 levels indicated that significantly elevated levels were observed in the presence of GSNO or STS (**Panels D - F**). The effects of 7 hr GSNO and STS treatment on ERK and P-ERK levels were assessed using western blot analysis (**Panels G - I**). GSNO treatment resulted in depletion of P-ERK in NT2 and hNP1 cells, but not in SH-SY5Y cells. STS treatment moderately inhibited P-ERK levels in all cell types. ERK levels did not appear to be altered as a result of GSNO or STS treatment.

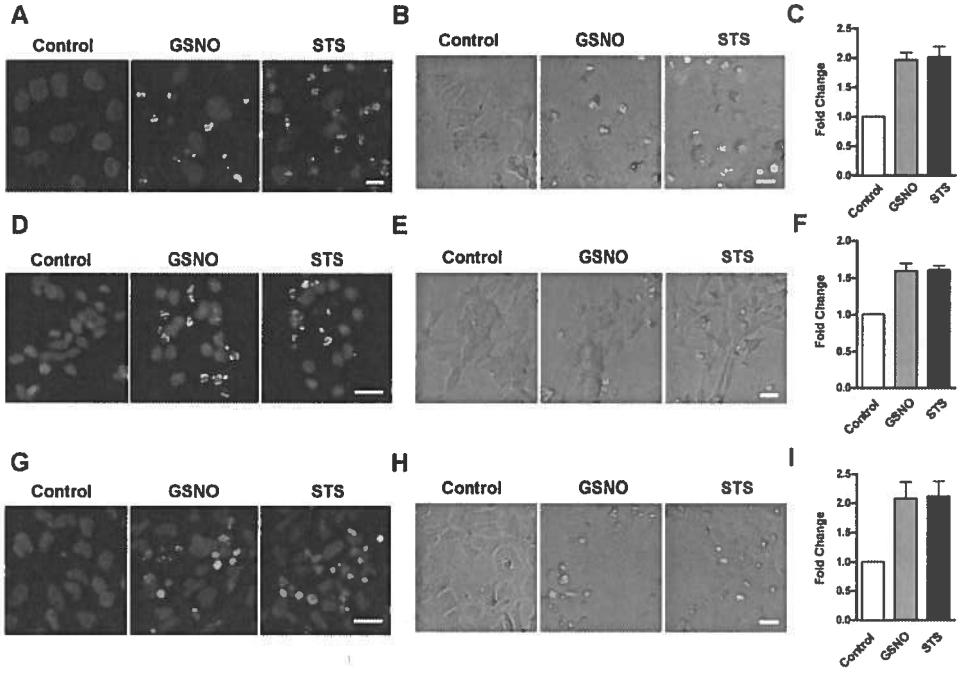
Figure 5. Analysis of the effects of GSNO and STS treatment on mitochondrial polarity. TMRM staining was assessed following 1, 4 and 22 hrs of GSNO or STS treatment. GSNO induced mitochondrial depolarization at 22 hrs in all cell types (Panels **A**, **B**, and **C**; NT2, SH-SY5Y, and hNP1 cells, respectively), but not at prior time points. Similarly, STS induced mitochondrial depolarization in all cells at 22 hrs. However, STS treatment produced mitochondrial hyperpolarization in NT2 and hNP1 cells at 1 and 4 hours, but not in SH-SY5Y cells. * $p < 0.05$, ** $p < 0.001$, **** $P < 0.0001$.

Figure 6. Schematic depiction of *S*-nitrosylation regulation of CASP3 mediated apoptosis and cell-type specific aspects of the induction of apoptosis by STS and GSNO. (**A**) Under normal conditions, the inhibitor of apoptosis proteins XIAP, which possesses E3 ubiquitin ligase activity, interacts with and ubiquitinates active caspase (CASP3). Ubiquitinated CASP3 is then degraded, thereby preventing its accumulation and participation in apoptosis. (**B**) In the presence of nitrosative stress (GSNO), both XIAP and CASP3 can undergo *S*-nitrosylation (SNO). XIAP E3 ligase activity is decreased upon *S*-nitrosylation, while SNO-CASP3 is capable of shedding its nitrosylation by transnitrosylating XIAP. The net effect of these events is to enable the accumulation of CASP3 and the development of apoptosis. (**C**) STS-induced apoptosis is characterized by cytochrome C (CytC) release from the mitochondria and the activation of CASP3. Based on our data, STS treatment increases CASP3 levels and apoptosis. In addition,

STS produces mitochondrial hyperpolarization ($\uparrow\Delta\Psi_m$) in hNP1, but not SH-SY5Y cells, suggesting that reactive oxygen species (ROS) are involved in STS-induced apoptosis in neural progenitors. While the induction of apoptosis by GSNO involves the potent activation of CASP3 altered ERK signalling is likely contributing to GSNO-induced apoptosis in NT2 and hNP1 cells, but not SH-SY5Y cells. Orange boxes indicate effects specific to neural progenitor cells.

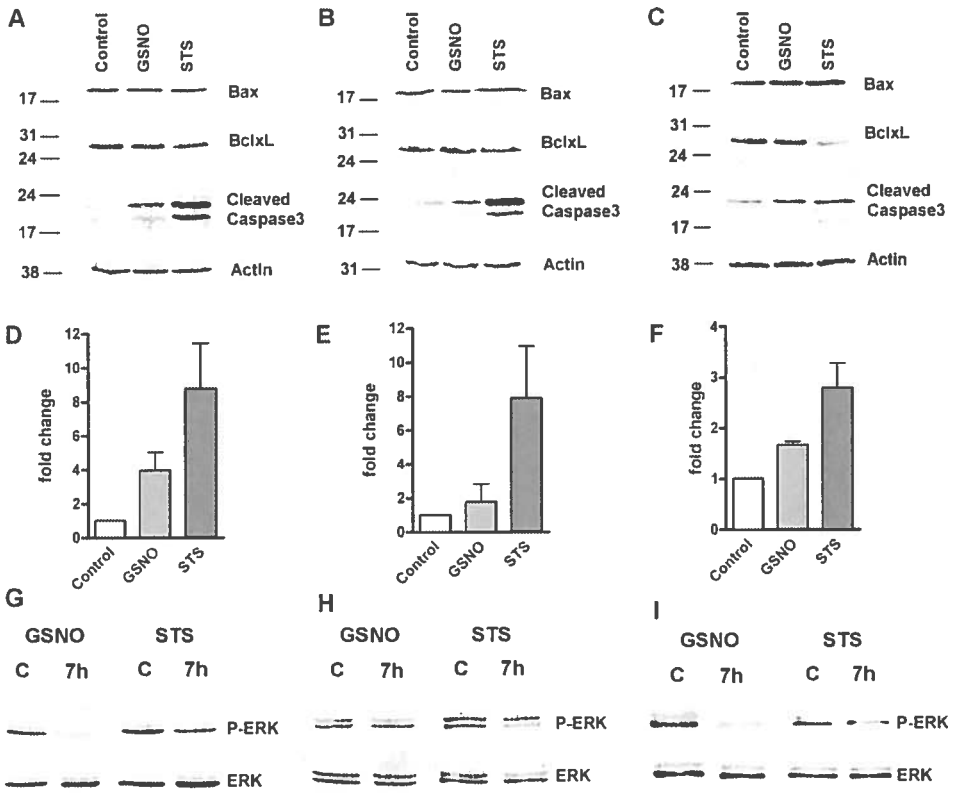


123x83mm (300 x 300 DPI)

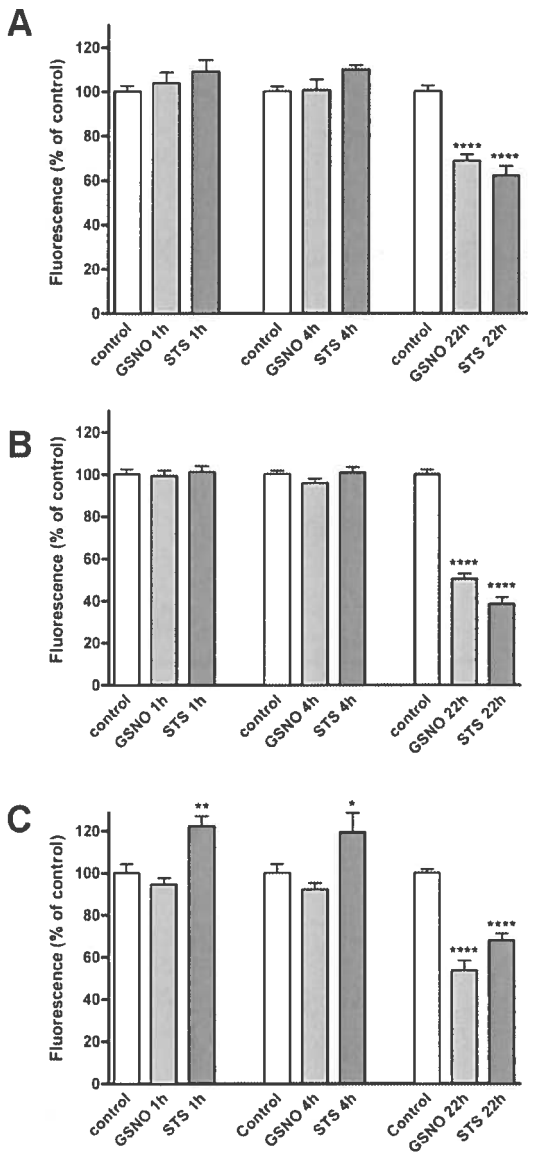


128x90mm (300 x 300 DPI)

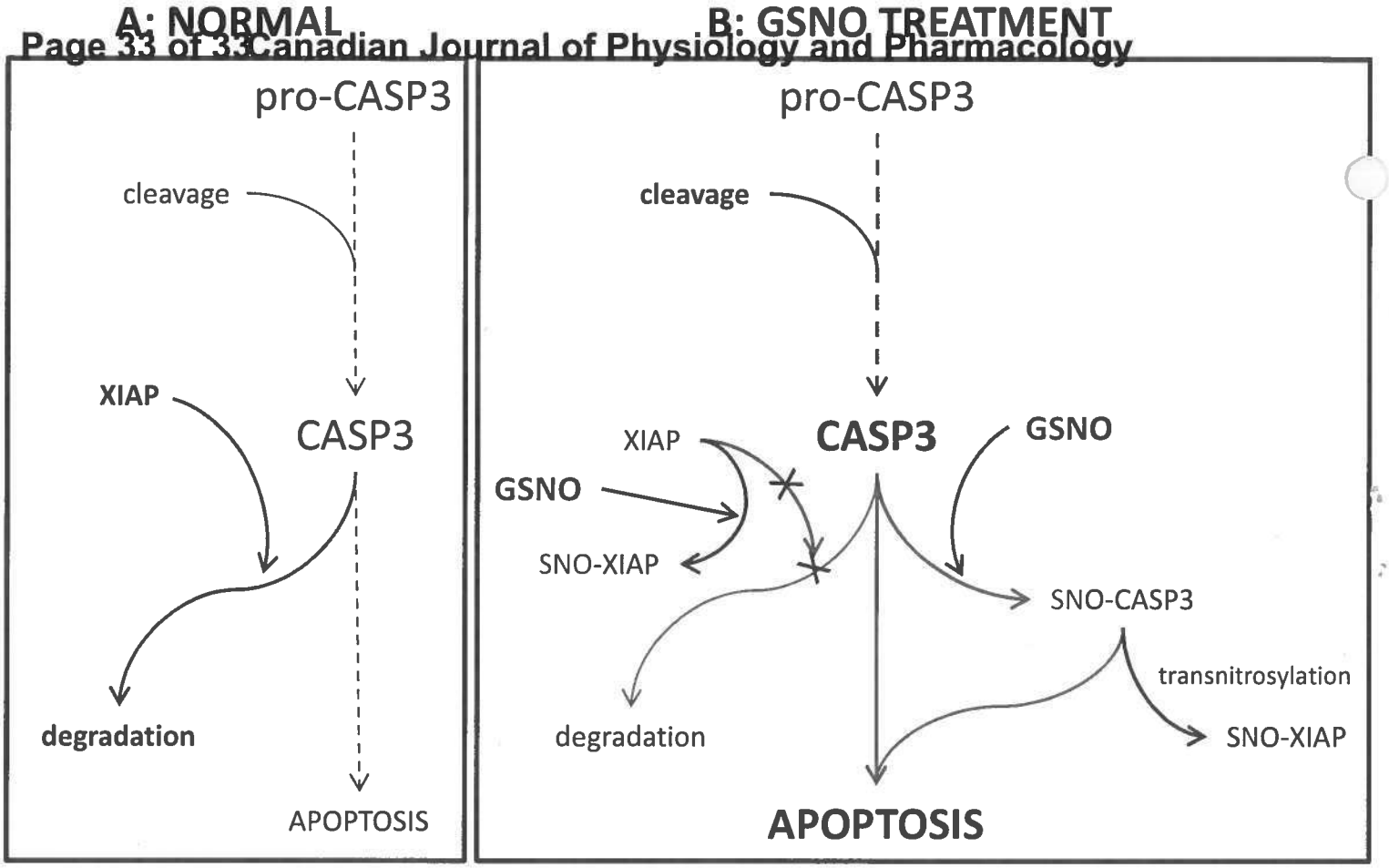
44x10mm (300 x 300 DPI)

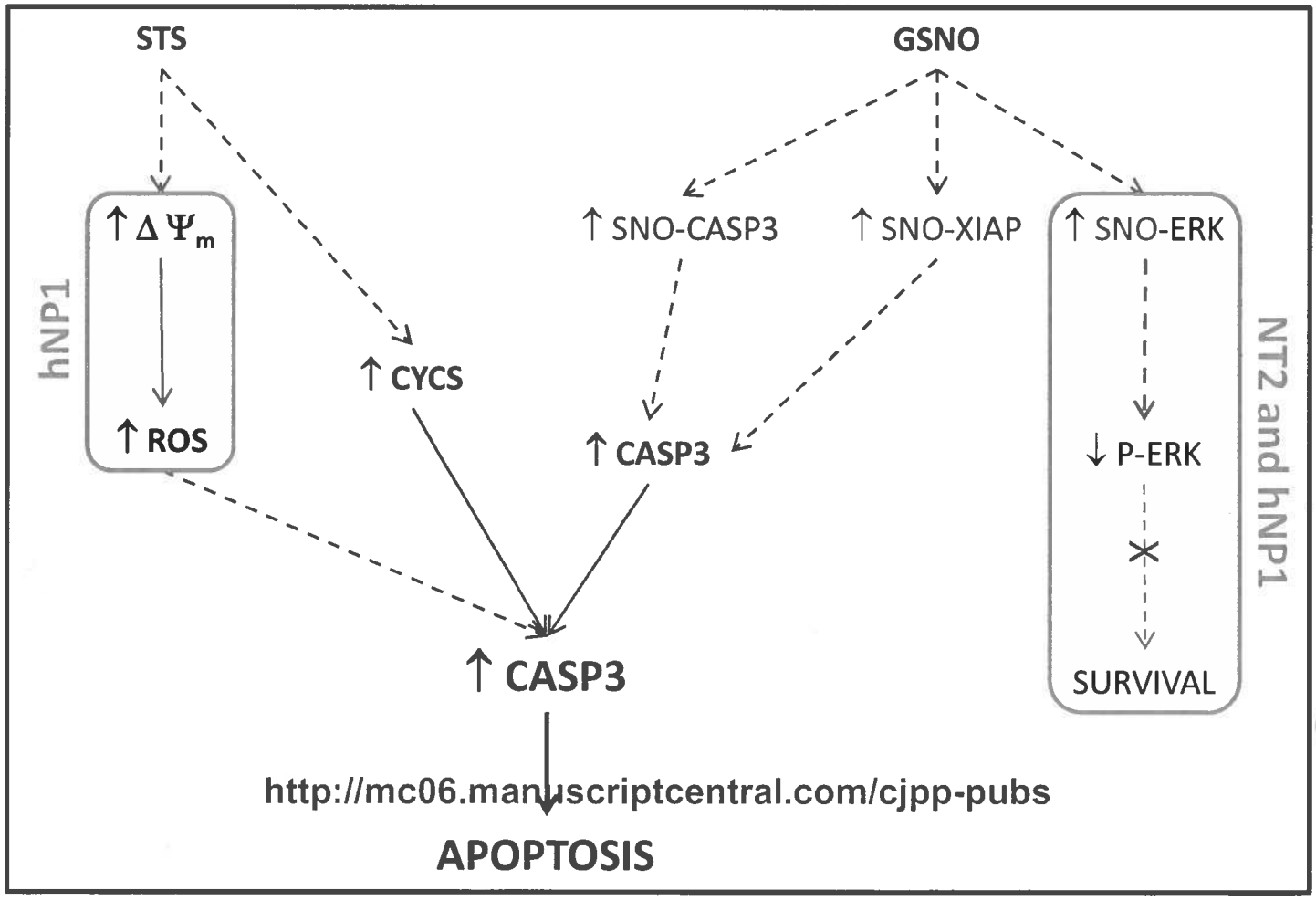


162x144mm (300 x 300 DPI)



178x370mm (300 x 300 DPI)


A: NORMAL
pro-CASP3
cleavage
XIAP
CASP3
degradation
APOPTOSIS
B: GSNO TREATMENT
pro-CASP3
cleavage
XIAP
CASP3
GSNO
GSNO
SNO-XIAP
SNO-CASP3
transnitrosylation
degradation
SNO-XIAP
APOPTOSIS


C: INDUCTION OF APOPTOSIS
STS
GSNO
↑ ΔΨm
↑ ROS
hNP1
↑ CYCS
↑ SNO-CASP3
↑ SNO-XIAP
↑ SNO-ERK
↑ CASP3
↓ P-ERK
SURVIVAL
NT2 and hNP1
↑ CASP3
APOPTOSIS