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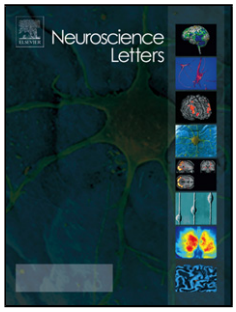
Journal Item



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Differing effects of NT-3 and GDNF on dissociated enteric ganglion cells exposed to hydrogen peroxide *in vitro*

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Highlights
Oxidative stress has been implicated in aging and disease-related damage of enteric neurons
Neurotrophic factors have been reported to have protective effects against oxidative stress
We assessed protective effects of NT-3 and GDNF in dissociated cultures of enteric ganglia
NT-3, but not GDNF protected against hydrogen peroxide-induced toxicity on enteric neurons

Abstract

Oxidative stress is widely recognized to contribute to neuronal death during various pathological conditions and aging. In the enteric nervous system (ENS), reactive oxygen species have been implicated in the mechanism of age-associated neuronal loss. The neurotrophic factors neurotrophin 3 (NT-3) and glial cell line-derived neurotrophic factor (GDNF) are important in the

development of enteric neurons and continue to be expressed in the gut throughout life. It has therefore been suggested that they may have a neuoprotective role in the ENS. We investigated the potential of NT-3 and GDNF to prevent death of enteric ganglion cells in dissociated cell culture after exposure to hydrogen peroxide (H_2O_2). H_2O_2 treatment resulted in a dose-dependent death of enteric neurons and glial cells, as demonstrated by MTS assay, Bis benzimide and propidium iodide staining and immunolabelling. Cultures treated with NT-3 prior to exposure showed reduced cell death compared to untreated control or GDNF-treated cultures. GDNF treatment did not affect neuronal survival in H_2O_2-treated cultures. These results suggest that NT-3 is able to enhance the survival of enteric ganglion cells exposed to oxidative stress.

Keywords: Neurotrophin-3, Glial cell line derived neurotrophic factor, enteric ganglia, oxidative stress, hydrogen peroxide

Introduction

The neurons of the enteric nervous system (ENS), located in the wall of the digestive tract, regulate intestinal functions, such as motility and secretion [4,6,13]. Neurodegenerative changes, including neuronal loss, have been described in the ENS during aging [7,27,30], and may contribute to gastrointestinal dysfunction, such as constipation and incontinence, which increase in incidence in the elderly [e.g.3]. The mechanisms underlying age-related enteric neuronal loss, however, are not understood, but there is evidence that reactive oxygen species (ROS) are elevated in myenteric neurons in old rats [37]. It has also been reported that myenteric neuronal loss is reduced in calorically-restricted rats[10,37].

Neurotrophic factors have been reported to protect neurons from oxidative stress [e.g.12,26,37,see 16,25]. It has therefore been suggested that increased survival of myenteric neurons in calorically-restricted animals might be due to the actions of neurotrophic factors present in the gut [37]. Two such factors, which continue to be expressed in the adult gut are neurotrophin 3 (NT-3) and glial cell line-derived neurotrophic factor (GDNF)[5,32]. Treatment of segments of intestinal smooth muscle (muscularis externa, in which myenteric ganglia are embedded) from calorically-restricted rats with NT-3 and GDNF reduced neuronal ROS levels and also enhanced resistance to menadione-induced apoptosis [37].

Here we examined the possible protective effects of NT-3 and GDNF in the ENS further, using a culture model of dissociated myenteric ganglion cells.

Possible protective effects of NT-3 and GDNF were examined under conditions of oxidative stress induced by hydrogen peroxide (H_2O_2), which is an established model [14,17,24] causing oxidative damage to cells *in vivo* [29]. Exogenous H_2O_2 readily enters cells [18] and induces apoptosis in many cell types [9]. H_2O_2 has also recently been shown to reduce numbers of vulnerable enteric neurons in an organotypic culture system [38]. Here we describe the effects of H_2O_2 treatment, in the presence and absence of NT-3 or GDNF, on dissociated myenteric ganglion cells from rat ileum.

2. Materials and Methods

2.1 Primary cultures of enteric ganglion calls and factor treatment

Dissociated cultures of isolated myenteric ganglia were used, to allow equivalent access of reagents to individual cells, and facilitate discrimination between individual cells when counting. Segments of myenteric ganglia were separated from muscularis externa of 7-day-old Sprague-Dawley rat ileum after incubation in collagenase type II (1mg/ml in HBSS containing 10μg/ml DNase) at 37°C. Ganglia were dissociated after 15 minutes incubation in trypsin-EDTA (Sigma) and passage through a 25 gauge needle. Cells were seeded in 150μl 199 medium with N1 supplements (199N1) containing 10% fetal calf serum (Sigma) at $2X10^4$ cells per 13mm glass coverslip coated with poly-L-lysine. After 1 hour incubation at 37°C, 2.5% CO_2, 850μl 199N1 medium was added. 16 hours later medium was replaced with serum-free 199N1. Cells were supplemented with desired concentrations of factors 24 hours after plating.

2.2 Hydrogen peroxide exposure

Dilutions of H_2O_2 (Sigma) were made fresh from 30% stock solution into HBSS for each experiment and was used at 1, 5, 10 and 25μM. Cultures of enteric ganglion cells grown with NT-3 or GDNF (10ng/ml) were exposed to H_2O_2 and subsequently incubated prior to the assay for 4 hours (for bis-benzimide/propidium iodide staining) or 6 hours (for MTS assay) at 37°C, 2.5% CO_2.

2.3 PGP 9.5 immunolabelling

Cultures were washed with phosphate buffered saline (PBS, pH7.3) and fixed with 4% paraformaldehyde for 1 hour at room temperature. Fixed cultures were incubated with primary rabbit anti-PGP 9.5 antibody (1:30000 in antibody diluting solution (ABDS:PBS, lysine, 0.l%sodium azide, 0.1%BSA, 1% Triton X-100) for 2 hours at room temperature, washed with PBS and incubated with fluorescein-conjugated goat anti-rabbit antibody (1:100 in ABDS) for one hour at room temperature. Cultures were washed with PBS and mounted on glass slides in Citifluor mountant.

2.4 Bis-benzimide/propidium iodide staining

Culture medium was replaced with 1ml of fresh medium. 10μl bis-benzimide (Hoechst stain, stock 500μg/ml in PBS) was added to each coverslip. After 20 minutes incubation at 37°C 10μl of propidium iodide (PI) was added and cultures were incubated at room temperature for 5 minutes. Subsequently all wells were washed two times with HBSS without phenol red (Sigma), fixed for1 hour in 4%

glutaraldehyde, washed in PBS and mounted on glass slides in Citifluor mountant.

2.5 Quantification of cell numbers

Cells stained with PGP9.5 or bis-benzimide and PI were counted under 400X magnification using either Zeiss Axiophot or Nikon Eclipse EB800 microscope. Bis-benzimide and PI-stained cells were counted in five random fields of view on each coverslip; PGP 9.5 stained cells were counted in a strip across the diameter of the coverslip. Data were obtained from three experiments and pooled for subsequent statistical analysis.

2.6 Viability assay

Viability of the cells exposed to H_2O_2 was assayed using CellTiter96^R kit (Promega), which allows colorimetric estimation of the number of viable cells. Briefly, growth medium of cultures exposed to H_2O_2 was replaced with HBSS without phenol red (Sigma), and MTS reagent was added to the cultures. After 4 hours incubation at 37°C absorbance was measured at 492nm.

2.7 Statistical analysis

Data were analysed by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test.

3. Results

3.1 Effects of NT-3 and GDNF on cultured enteric ganglion cells

Preliminary experiments were performed to assess the effects of NT-3 and GDNF in our culture model and to examine the extent of cell death occurring naturally during culture. Cultures containing enteric neurons and glial cells were

grown with 10ng/ml NT-3 or GDNF for 12 and 36 hours. Immunolabelling of cultures containing both neurons and glial cells with the neuronal marker PGP9.5 allows clear distinction between the two cell types, enabling accurate cell counts. No significant change in neuronal number was measured after 12 hours treatment with either NT-3 or GDNF (Figure 1). Glial cell numbers were also similar in control, NT-3- and GDNF-treated cultures after 12 hours (data not shown). After 36 hours incubation, control cultures showed a significant ($p<0.01$) decline in neuronal numbers. In contrast, cultures grown with NT-3 exhibited reduced neuronal death compared to controls ($p<0.01$). Interestingly, GDNF treatment stimulated a marked increase ($p<0.01$) of neuronal numbers, which exceeded those found in cultures treated with GDNF for 12 hours. Glial cell numbers were affected by GDNF in a manner similar to neurons, whereas glial cell numbers in NT-3-treated cultures were not different than those found in controls (data not shown).

These results confirm that both NT-3 and GDNF are able to affect the behaviour of enteric ganglion cells in this culture system. In order to minimize effects of cell death observed in cultures grown for 36 hours on the results of survival assays, further experiments were restricted to the 12 hour time point.

3.2 Effects of different concentrations of H_2O_2 on enteric ganglion cells in culture

Pilot experiments were performed to determine the response of enteric ganglion cells to different concentrations of H_2O_2. Cultures were exposed to 1,5,10 or 25μM H_2O_2 then incubated for 6 hours before assessing cell viability by MTS assay. Effects of H_2O_2 on the viability of enteric ganglion cells are shown in

Figure 2. While 1μM H_2O_2 did not significantly affect cell viability, higher concentrations decreased it in a significant dose-dependent manner, with about 50% decrease in viability at 10μM H_2O_2 compared to control. 25μM H_2O_2 eliminated almost all cells, therefore for further experiments H_2O_2 was used at 1, 5 and 10 μM.

Analysis of PGP9.5-immunolabelled cultures treated in the same way as those used for MTS assay revealed that neurons and glial cells were differentially affected by H_2O_2 (Table 1). Neurons were more vulnerable to H_2O_2 than glial cells.

3.3 Effects of pre-treatment with neurotrophic factors on viability of H_2O_2-treated enteric ganglion cells

After optimising culture time and H_2O_2 concentrations, we investigated whether treatment of enteric neurons with 10ng/ml NT-3 or GDNF prior to H_2O_2 exposure increased viability of enteric ganglion cells. Cultures grown with NT-3 exhibited a slightly increased survival compared to controls, although the difference was not statistically significant at any H_2O_2 concentration (Figure 3A); GDNF treatment did not affect cell viability (Figure 3B).

The effects of factors were then examined in more detail, employing bis-benzimide and PI (live/dead) staining; bis benzimide stains nuclei of all cells, while PI is excluded from living cells, therefore allowing accurate measure of total cell numbers and the extent of cell survival. Cell cultures were incubated for 12 hours with 10ng/ml NT-3 or GDNF, then treated with 10μM H_2O_2 and stained with PI and bis-benzimide 4 hours later. This shorter H_2O_2 exposure time resulted in

reduced cell losses compared to those seen after 6 hours exposure (Table 1). H_2O_2-treatment of control cultures without trophic factors resulted in a significant increase in PI-positive cells ($p<0.02$, Figure 4A). Cultures grown with NT-3 prior to H_2O_2 exposure exhibited significantly ($p<0.01$) decreased numbers of PI-stained cells compared to H_2O_2 controls without factor treatment. GDNF treatment also decreased PI positive cells in cultures exposed to H_2O_2, but the effect was not statistically significant. Cultures not exposed to H_2O_2 showed comparable numbers of PI stained cells in each factor treatment and in controls.

Counts of Hoechst positive cells are summarized in Figure 4B. In the absence of H_2O_2, NT-3 did not affect total cell numbers, but GDNF increased cell numbers compared to both control ($p<0.05$) and NT-3-treated cultures ($p <0.02$). This observation confirms the results of the cell counts performed on PGP9.5 immunolabelled cultures. Addition of H_2O_2 had no effect on cell numbers in untreated or NT-3-treated cultures (Hoescht labels both live and dead cells), but interestingly decreased the total cell numbers in cultures grown with GDNF ($p=0.01$), reducing cell numbers to levels similar to those in control and NT-3-treated cultures exposed to H_2O_2.

4. Discussion

In this study, we investigated the effects of NT-3 and GDNF on cultured enteric ganglion cells exposed to H_2O_2. Our results demonstrate decreased cell death in cultures grown with NT-3 prior to H_2O_2 exposure, as opposed to control cultures, and cultures grown with GDNF. In contrast, GDNF stimulated an

increase in the number of cells present in the cultures, an effect abrogated by H_2O_2 treatment.

Two previous studies have demonstrated that H_2O_2 has a toxic effect on enteric neurons *in vitro*. These studies either used an organotypic model of rat myenteric ganglia, in which the ganglia remained intact [1,38] or a mixed preparation of cells from dissociated whole intestine from embryonic mice [1]. The cultures in the latter study were manipulated to remove enteric glia, which were thereby shown to have a protective effect against H_2O_2-induced neuronal toxicity [1]. The protective effects of NT-3 in the present study were unlikely to be due to an increased number of enteric glia in NT-3-treated cultures, because glial cell numbers were not significantly different between the NT-3-treated cultures and the control or GDNF-treated cultures. Moreover in previous work, in which dissociated enteric ganglia were grown in the presence of NT-3 for longer periods, glial numbers were not increased [31]. In future studies, however, counts of neurons and glia in H_2O_2-treated cultures should be performed.

NT-3, a member of the neurotrophin family, promotes differentiation of enteric neurons and glia from precursor cells during ENS development [5,32]. NT-3 has also been found to protect against menadione-induced apoptosis of myenteric neurons in the muscularis externa from calorically-restricted rats [37]. The present results are thus in keeping with this previous report. Other members of the neurotrophin family, notably BDNF, have been found to be protective against oxidative stress in several different neuronal culture systems [36,16,25].

GDNF is a member of the GDNF family of neurotrophic factors. It has been shown to perform a critical role in the survival, as well as proliferation of ENS precursor cells [5,32] and has previously reported to protect against oxidative stress in isolated intestinal preparations [37] and some other systems [e.g.26], and against hyperglycaemia –induced myenteric neuronal death in culture [2]. Here we found that GDNF did not prevent H_2O_2-induced cell death in cultures of enteric ganglion cells, under the conditions employed. We did observe however, that GDNF treatment increased the total number of cells in the cultures, as shown by the results of cell counts of both PGP9.5 and Hoechst stained cultures. Postnatal enteric ganglia contain neural precursors [33,34], so it is not unexpected that GDNF would promote differentiation of these cells *in vitro*. GDNF is known to have a number of actions in the postnatal ENS [28]. Interestingly, the present results suggest that new cells arising due to GDNF treatment may be more vulnerable to oxidative damage than differentiated neurons and glia present in the cultures. This possibility is suggested by the reduction of total cell numbers measured in GDNF-treated cultures exposed to H_2O_2, that reached levels found in non-factor treated controls. One possible explanation of this finding is differing sensitivities of mature and dividing cells to DNA-damaging agents, such as oxidative stress; immature cells undergoing apoptosis rather than activating DNA repair and cell survival programs [20,24].

Our findings raise the question of the mechanisms involved in the protective effect of NT-3 on enteric ganglion cells. One possible explanation is that NT-3 might influence protein levels or activity of antioxidant enzymes [23].

Evidence supporting this possibility includes observations that nerve growth factor stimulates activity of glutathione peroxidase [11] and BDNF exerts protective effects on auditory neurons via increased levels of glutathione [15]. Mattson et al. [23] demonstrated that neurotrophic factors increased antioxidant enzyme activity in cultured hippocampal neurons. Neurotrophic factor treatment could also lead to reduced ROS generation; NT-3 treatment decreased generation of free radicals by myenteric neurons in muscularis externa preparations [37]. Finally, it is known that neurotrophic factors activate signalling pathways that promote expression of other survival-promoting proteins [e.g.29].

In conclusion, these results provide further support for the suggestion that neurotrophic factors, particularly NT-3, may have protective roles in the ENS. Such an effect could have important implications not just for aging, but also disease states such as diabetes, which affect the ENS and gastrointestinal functions [e.g.8]. In this context, it is important to note that oxidative stress has been implicated in enteric diabetic neuropathy [8,19,38] and that recent evidence has shown a reduction in the levels of neurotrophic factors in the diabetic gut [21].

Acknowledgements:

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Figure Legends:

Figure 1: Mean cell numbers in cultures after 12 and 36 hours incubation with NT-3 or GDNF (10ng/ml). Data from 3 separate experiments; 3 replicas per experiment. Error bars represent ±S.E.M. *** p<0.001;**p<0.01

Figure 2: Survival of cultured enteric ganglion cells exposed to different concentrations of H_2O_2, measured using MTS assay. Results are expressed as percentage of untreated controls. Data are means of 3 separate experiments; 4 replicas per experiment. Error bars represent ±S.E.M. ***p<0.001

Figure 3: Survival of enteric ganglion cells treated with 10ng/ml NT-3 (**A**) or GDNF (**B**) prior to H_2O_2 exposure. Means from 3 separate experiments, expressed as a percentage of untreated controls; 4 replicas per experiment. Error bars represent ±S.E.M.

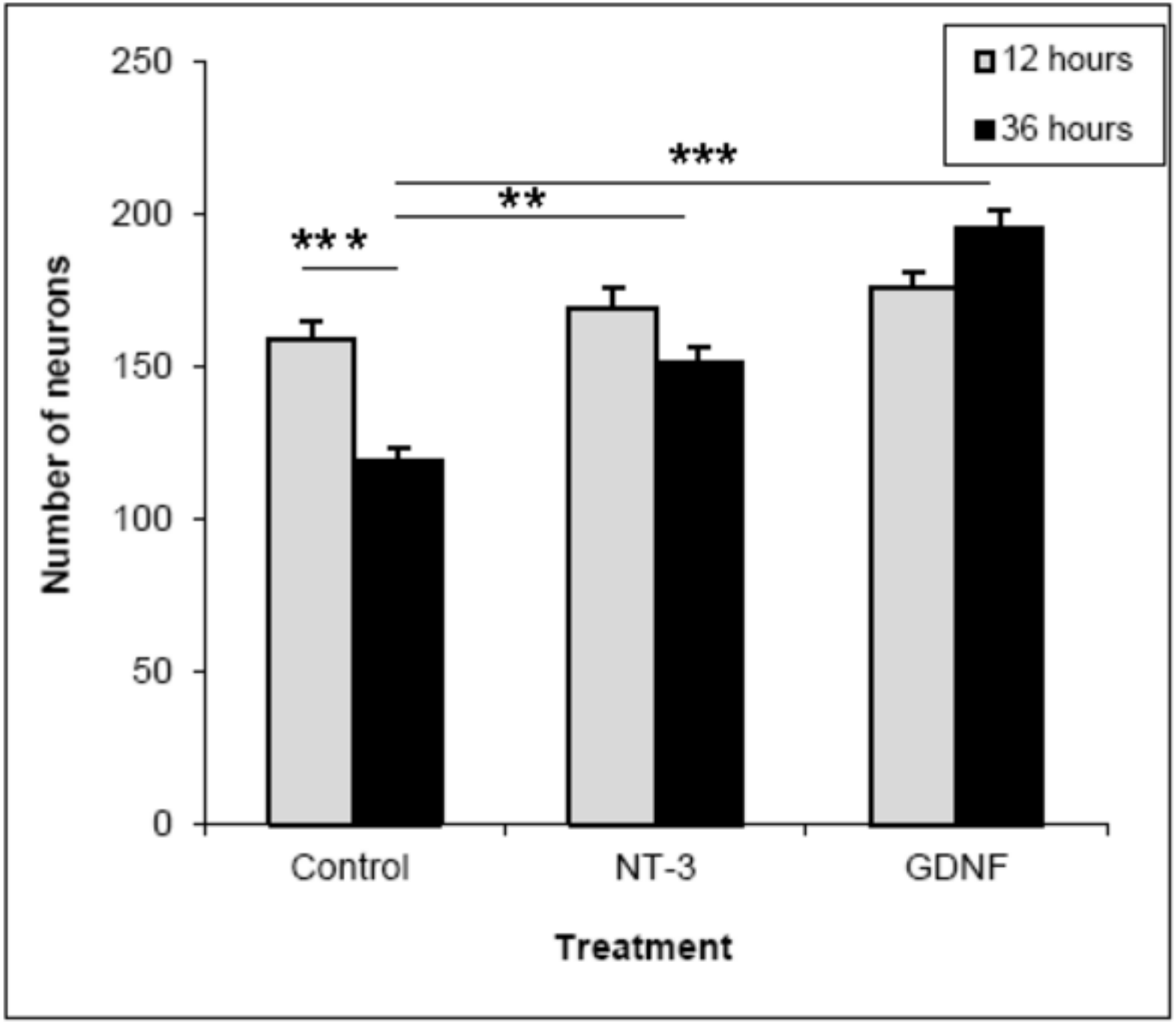
Figure 4: Total number of PI (**A**) and Hoechst (**B**) positive cells in NT-3 and GDNF (10ng/ml) treated cultures after exposure to H_2O_2, compared to controls. Graphs show mean number of stained cells counted in 3 separate experiments; 3 replicas per experiment. Error bars represent ±S.E.M.

***p≤0.01;**p<0.02; *p<=0.05

Table 1: Effects of H_2O_2 on neuronal and glial cell numbers

Treatment	**Number of neurons (± SEM)**	**Number of glial cells (± SEM)**	**N/GC ratio**
Control	440 ± 9.3	912 ± 14.6	0.48
5 μM H_2O_2	129 ±13.9	404 ±33.6	0.32
10 μM H_2O_2	15 ± 1	88 ± 5.1	0.17
25 μM H_2O_2	1	15 ± 2.6	0.06

Revised Figure 1



Revised Figure 2