

## Synergistic Interactions between Commonly Used Food Additives in a Developmental Neurotoxicity Test

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Exposure to non-nutritional food additives during the critical development window has been implicated in the induction and severity of behavioral disorders such as attention deficit hyperactivity disorder (ADHD). Although the use of single food additives at their regulated concentrations is believed to be relatively safe in terms of neuronal development, their combined effects remain unclear. We therefore examined the neurotoxic effects of four common food additives in combinations of two (Brilliant Blue and L-glutamic acid, Quinoline Yellow and aspartame) to assess potential interactions. Mouse NB2a neuroblastoma cells were induced to differentiate and grow neurites in the presence of additives. After 24 h, cells were fixed and stained and neurite length measured by light microscopy with computerized image analysis. Neurotoxicity was measured as an inhibition of neurite outgrowth. Two independent models were used to analyze combination effects: effect additivity and dose additivity. Significant synergy was observed between combinations of Brilliant Blue with L-glutamic acid, and Quinoline Yellow with aspartame, in both models. Involvement of N-methyl-D-aspartate (NMDA) receptors in food additive-induced neurite inhibition was assessed with a NMDA antagonist, CNS-1102. L-glutamic acid- and aspartame-induced neurotoxicity was reduced in the presence of CNS-1102; however, the antagonist did not prevent food color-induced neurotoxicity. Theoretical exposure to additives was calculated based on analysis of content in foodstuff, and estimated percentage absorption from the gut. Inhibition of neurite outgrowth was found at concentrations of additives theoretically achievable in plasma by ingestion of a typical snack and drink. In addition, Trypan Blue dye exclusion was used to evaluate the cellular toxicity of food additives on cell viability of NB2a cells; both combinations had a straightforward additive effect on cytotoxicity. These data have implications for the cellular effects of common chemical entities ingested individually and in combination.

**Key Words:** neurotoxicity; *in vitro*; food additives; synergy; interactions.

Exposure to non-nutritional food additives during the critical development window, which in humans extends from the sixth month of gestation to several years after birth, has been implicated in the induction and severity of some childhood behavioral and developmental disorders, such as attention deficit hyperactivity disorder (ADHD) (Boris and Mandel, 1994; Feingold, 1975). The causation of this condition by food additives is somewhat controversial, although a number of controlled clinical trials that have eliminated food colorings, preservatives, and flavor enhancers from the diets of hyperactive children have shown an improvement in behavior rating, learning test performance, and health (Bateman *et al.*, 2004; Egger *et al.*, 1985; Swanson and Kinsbourne, 1980), whereas other studies disprove this relationship (Gross *et al.*, 1987; Harley *et al.*, 1978).

The safety of two flavor enhancers—L-glutamic acid (monosodium glutamate) and aspartame (1-methyl N-L-alpha-aspartyl-phenylalanine)—has been examined extensively, and concerns have been expressed over their excitotoxic effects, i.e., their potential for destroying central neurons by excessive stimulation of postsynaptic excitatory membrane receptors, predominantly the NMDA subtype of glutamate receptor (Olney, 1988, Rothman and Olney, 1995). Several studies have shown that these excitotoxins, when administered to neonatal animals, produce acute neuronal degeneration in the retina and in specialized regions of the brain: the circumventricular organs that lack blood brain barriers (Olney, 1969a,b; Olney and Ho, 1970; Olney *et al.*, 1972).

Although acceptable daily intake values (ADI) have been derived from the safety assessment of each food additive, their combined adverse effects are unclear and have not been widely studied. Food additives are typically used in combination within processed foods and therefore collectively may have some adverse effects at the cellular level, even if their individual concentrations are below the recommended ADI value. By determining if combinations of such additives modify the functions of individual neurons, one might gain a better appreciation of their potential to modify more complex neurological systems. Furthermore, it is important to determine

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the effects of food additives in model systems that mimic neuronal development within the critical development window in neonatal humans.

The differentiation of neurons in culture (seen as neurite outgrowth) is a physiological process that is a general indicator of cellular well being. Its measurement therefore provides a useful *in vitro* model for the assessment of neurotoxicity, and has been successfully used to demonstrate the neurotoxic potential of a wide range of agents (Flaskos *et al.*, 1998; McLean *et al.*, 1998; Smith *et al.*, 1997), including excitatory amino acids (Abdulla and Campbell, 1993). Neurite outgrowth is a specific structural end-point unique to the nervous system and depends upon a number of critical cellular processes such as axonal transport. The inhibition of neurite outgrowth is only one marker of neurotoxicity that involves differentiating cells; thus, it may be of more relevance to exposure of the developing nervous system, rather than the mature nervous system. The mouse NB2a neuroblastoma cell has been determined to be a sensitive predictor of neurotoxicity (Axelrad *et al.*, 2002; Flaskos *et al.*, 1998; McLean *et al.*, 1998) and its relative ease of culture and reproducibility suggested it was suitable for further use and development of experiments involving the assessment of interactions.

Assessing the combined effect of chemicals is extremely complex and often unpredictable; thus all possible effects resulting from interactions need to be considered when assessing risk from exposure to chemicals. These effects include: additivity, where agents are no more and no less effective in combination than they are separately; synergism, where the effectiveness of agents is increased when in combination; potentiation, where the increased effect of a toxic compound is acting concurrently with a non-toxic compound; and finally, antagonism, where the effectiveness of agents is decreased when in combination. There is ongoing discussion regarding the most appropriate method for the evaluation of interactions (Berenbaum, 1989; Kortenkamp and Altenburger, 1998); therefore two methods have been employed in this study: the "effect additivity" model (Axelrad *et al.*, 2002; Bliss, 1939) and the alternative "dose additivity" model (Berenbaum, 1978; Loewe, 1953).

Four common food additives (Brilliant Blue and L-glutamic acid, Quinoline Yellow and aspartame) were chosen for this study to determine whether they cause neurotoxicity, measured by their role in inhibiting neurite outgrowth from differentiating neuroblastoma cells. Individual additives were used at concentrations that alone produce little or no neurotoxicity to evaluate whether they interact with one another when combined.

## MATERIALS AND METHODS

**Cell line and reagents.** Mouse NB2a neuroblastoma cells (cell line: 89121404) were obtained from ECACC (Salisbury, U.K.). DMEM culture

media and foetal calf serum were purchased from Gibco BRL Life Technologies (Uxbridge, U.K.), and tissue culture plastic-ware was from Nunc/ Fisher Scientific (Loughborough, U.K.). Aspartame, Quinoline Yellow, and Trypan Blue were obtained from ICN Pharmaceuticals, Ltd. (Basingstoke, U.K.). Brilliant Blue was supplied by Neelikon food dyes & chemicals Ltd. (Middlesex, U.K.). L-glutamic acid, CNS-1102 (Aptiganel hydrochloride), dibutyryl cyclic AMP, phosphate-buffered saline, horse serum, penicillin/streptomycin solution, and gentamicin were supplied by Sigma (Poole, U.K.).

**Cell culture.** The NB2a cell line was maintained at 37°C in a controlled humidified incubator in 5% CO<sub>2</sub>. The proliferation medium consisted of high glucose Dulbecco's Modified Eagle's Medium (DMEM) containing Glutamax-1, and supplemented with 5% (v/v) FCS, 5% (v/v) HS, 100 U/ml penicillin plus 100 µg/ml streptomycin, and 25 µg/ml gentamicin.

**Measurement of neurite outgrowth.** NB2a neuroblastoma cells were plated in the above proliferation medium on to 48-well plastic culture plates at a density of 15,000 cells/ml. After 24 h, the cells had adhered to the plate, and were then induced to differentiate by removal of serum and addition of 0.5 mmol.l<sup>-1</sup> dibutyryl cyclic AMP. Differentiation led to the growth of neurite-like extensions from the neuronal cell body (referred to as neurites for the purpose of this study). Differentiating cells were exposed to food additives at various concentrations for a further 24 h. Cells were then fixed in 4% w/v formaldehyde and stained with Coomassie Blue stain (0.6% (w/v) Coomassie Brilliant Blue G in 10% (v/v) acetic acid, 10% (v/v) methanol in PBS).

All processes formed following differentiation including axon-like processes (defined as extensions greater than two cell body diameters in length with an extension foot), as well as those less than two cell body diameters were considered to be neurites. Total neurite length (µm) per cell was measured by light microscopy with computerized image analysis using a Zeiss Axiovert 35 M microscope linked by a video camera to a Neurite Outgrowth Explora Nova 1.00a image analyzer (La Rochelle, France). All treatments were performed in triplicate; a minimum of 200 cells from approximately 10 different fields over 3 wells were viewed and analyzed. The automated Neurite Outgrowth Explora Nova software performed a segmentation of the acquired images of neurons: detection and subtraction of cell bodies, followed by the skeletalization of the remaining neurites by serial erosion to single pixel width. Average total neurite length per cell for each treatment was recorded. Neurotoxicity was measured as an inhibition in neurite length (i.e., % inhibition of neurite outgrowth) for treated cells compared to differentiating controls, with a base-line level of zero neurite outgrowth measured from negative control cells that were not induced to differentiate.

**Assessment of interactions using the effect method—"effect additivity".** In order to permit detection of either inhibition or potentiation of neurite outgrowth, cells were induced to differentiate with either a single additive at a concentration that led to 20–25% inhibition of neurite outgrowth or with a mixture calculated to produce the same effect of 20–25% inhibition if their effects were simply additive (i.e., zero interaction). Cells were exposed to pairs of the compounds in the following proportions: 100% A: 0% B, 75% A: 25% B, 50% A: 50% B, 25% A: 75% B, and 0% A: 100% B. Each such experiment was performed on four independent occasions.

In this model of synergy (Axelrad *et al.*, 2002; Bliss, 1939), the combined effect of two agents is thought to be equal to the sum of the effects of the single compounds; thus the inhibition of neurite outgrowth produced by any mixture can be predicted. Deviations from this expected constant inhibition (zero interaction) produced by the calculated IC<sub>20–25</sub> are either synergistic (above expected zero effect), or antagonistic (below expected zero effect). Statistical analysis was calculated by Student's two-tailed *t*-test. *p* values of <0.05 were considered to be significant.

**Assessment of interactions using the isobole method—"dose additivity".** NB2a neuroblastoma cells were induced to differentiate in the presence of mixtures of compounds at various concentrations, chosen to be below those producing maximum neurite inhibition. Concentrations for each additive used (four concentrations of colours and six concentrations of flavour

enhancers) ranged from 0.05–500 nM of Brilliant Blue, with 0.5–100  $\mu$ M of L-glutamic acid, and 1–50  $\mu$ M of Quinoline Yellow, with 0.5–100  $\mu$ M of aspartame, i.e., exposure to 24 treatments per combination. Concentration-response curves for the mixtures (flavor enhancers in the presence of a fixed concentration of a color) were then produced, and concentrations of individual additives within a mixture required to inhibit neurite outgrowth by 50% ( $IC_{50}$ ) were obtained from regression analysis. Analysis of neurite outgrowth with each combination was performed in four independent experiments.

The isobole model (Berenbaum, 1978) allows the construction of graphs showing curves describing various combinations of two compounds A and B, which together produces the same, specified effect. Iso-effective doses A and B of the single compounds are connected by an additivity line, which predicts the combinations of A and B required to yield the specified effect, provided the interaction between A and B is additive (zero interaction). This relationship is expressed by the equation:

$$c_A/C_A + c_B/C_B = 1 \quad (1)$$

where  $c_A$  and  $c_B$  are the concentrations of A and B in a mixture that produce a specified effect, and  $C_A$  and  $C_B$  are the concentrations of the single agents, which on their own elicit the same effect as the mixture. Synergistic agents require lower concentrations to produce a given effect when in combination, giving concave isoboles; therefore the equation is expressed as  $c_A/C_A + c_B/C_B < 1$ . Antagonistic combinations give convex isoboles resulting in  $c_A/C_A + c_B/C_B > 1$ .

For statistical analysis, the total concentration of a mixture producing 50% neurite inhibition, denoted  $(IC_{50})_{mixture}$ , must be compared to the corresponding value calculated for a theoretically additive total concentration, denoted  $(IC_{50})_{additive}$ . Regression lines of total concentration were used to calculate  $(IC_{50})_{mixture}$  and its standard error. Since compound B remains constant throughout a test in which varying concentrations of compound A are added, individual concentration-response data from compound A and the known constant concentration of compound B can be used to calculate  $(IC_{50})_{additive}$  and its standard error (square root of variance) using equations from Tallarida (1992).

$$(IC_{50})_{add} = C_A - R(c_B) \quad (2)$$

$$V(IC_{50})_{add} = V(C_A) + (c_B)^2 V(R) - 2c_B V(C_A) / C_B \quad (3)$$

where  $c_B$  is a fixed concentration of compound B,  $R$  is the relative potency ratio at that effect level, and  $V(R)$  is its variance given by:

$$R = (C_A / C_B)$$

$$V(R) = V(C_A) / (C_B)^2 + (C_A)^2 V(C_B) / (C_B)^4$$

The differences between the respective additive and experimentally determined values were analysed statistically using Student's *t*-test on the logarithmic scale. *p* values of <0.05 were considered to be significant.

**Role of NMDA receptors in food additive-induced neurotoxicity.** Involvement of N-methyl-D-aspartate (NMDA) receptors in food additive-induced neurite inhibition was assessed by the use of CNS-1102, a non-competitive NMDA receptor antagonist. NB2a cells were plated on to 48-well plastic culture plates at a density of 15000 cells/ml. After 24 h, cells were then induced to differentiate for a further 24 h in the presence of individual food additives at concentrations known to produce approximately 70% neurite inhibition ( $IC_{70}$ ): 8.99  $\mu$ M Brilliant Blue, 5.49 mM Quinoline Yellow, 838.6  $\mu$ M L-glutamic acid, and 9.61 mM aspartame. CNS-1102 (0.1–10  $\mu$ M) was added into culture medium 30 min before treatment with additive. Cells were then fixed and stained, and neurite length measured as previously described. Statistical analysis was calculated by Student's two-tailed *t*-test. *p* values of <0.05 were considered to be significant.

**Cytotoxicity and cell viability.** Cell viability was assessed by Trypan Blue dye exclusion. NB2a cells were plated on to 6-well plates at a density of

$5 \times 10^4$  cells per well in serum-containing medium at a volume of 1500  $\mu$ l. Cells were then induced to differentiate in the presence of individual additives (0.05 nM of Brilliant Blue, 0.5  $\mu$ M of L-glutamic acid, Quinoline Yellow, and aspartame), or mixtures of the combinations previously studied. The effect additivity model was used to analyze interactions. After 24 h treatment, 20  $\mu$ l Trypan Blue was added to 100  $\mu$ l cell suspension. Cells were loaded on a haemocytometer; stained and unstained (viable) cells were observed in a Zeiss Axiovert 35 M microscope, and the mean percentage of dead cells was calculated. Statistical analysis was calculated by Mann-Whitney U test. *p* values of <0.05 were considered to be significant.

**Analysis of food additive content in foodstuff.** Five products were analysed for specific additive content. Colors Brilliant Blue and Quinoline Yellow were analyzed by RSSL (Reading, U.K.), whilst flavor enhancer L-glutamic acid and sweetener aspartame were analyzed by Microsearch (West Yorkshire, U.K.). Both colors were extracted by standard methodology with methanolic ammonia and analyzed by reverse-phase HPLC with diode array detection. Aspartame was analyzed by HPLC with UV detection, and L-glutamic acid by HPLC with Refractive Index detector. All samples were analyzed in the form they were available for consumption. Theoretical exposure to additives was calculated based on this analysis of food additive content, and estimated percentage absorption from the gut ( $\approx$ 10% for Brilliant Blue,  $\approx$ 6% for Quinoline Yellow, and 100% for both aspartame and L-glutamic acid) (JECFA, 1970, 1981, 1984, 1988) following ingestion of the products by a 10 kg child. The concentrations of compounds in mg/l were converted into molar concentration using the molecular weight of the product in the form in which it was tested in the *in vitro* neurotoxicity assay.

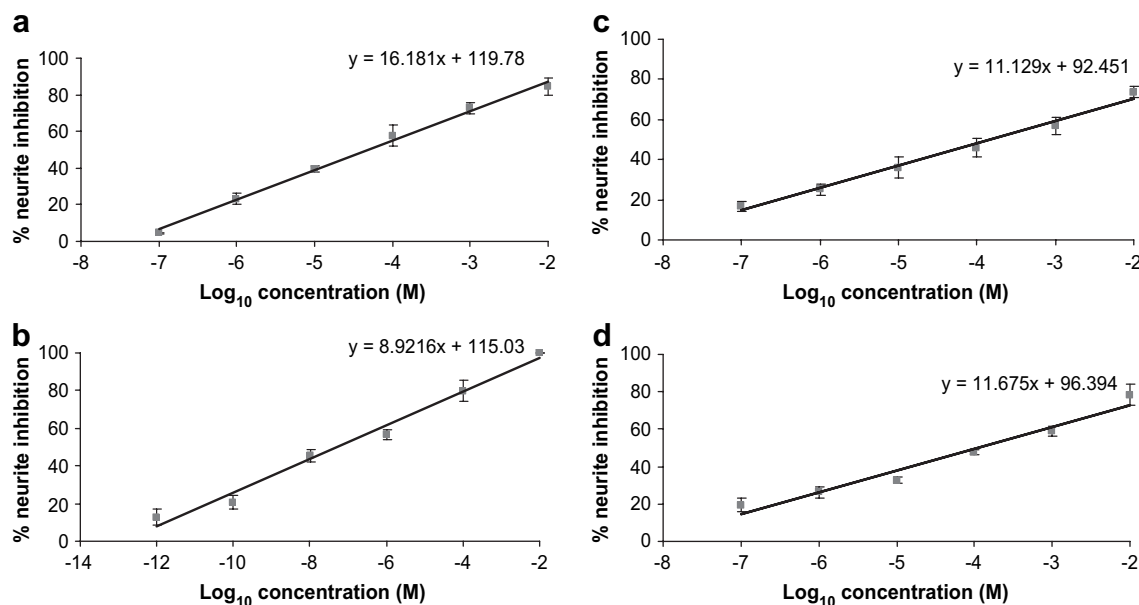
## RESULTS

### Determination of $IC_{20-25}$ and $IC_{50}$ Values for Individual Compounds

The average neurite length produced by NB2a cells in the presence of various concentrations of individual additives ( $n = 4$ ) was determined. Mean percentage neurite inhibition was calculated at each concentration and a log concentration-response curve produced (Fig. 1). Brilliant Blue was found to be the most potent of the additives (Mean  $IC_{50}$  (SEM) = 51.4 (21.2) nM), followed by L-glutamic acid ( $IC_{50} = 48.7$  (9.7)  $\mu$ M), Quinoline Yellow ( $IC_{50} = 106$  (31.8)  $\mu$ M) and aspartame, being the least potent of the four ( $IC_{50} = 153$  (51.5)  $\mu$ M).

### Assessment of Synergistic Neurotoxicity of Food Additive Combinations: Measurement of Interactions by "Effect Additivity"

In the absence of interactions, it would be expected that two compounds in combinations at fixed concentrations would have the same effect as the individual compounds at those same concentrations; this was taken as the "theoretical expected values." To determine if this assumption was appropriate, i.e., that the expected effect of two agents was actually equal to the sum of the effects of the single compounds, separate experiments were carried out in which cells were treated with single compounds at 50% of the  $IC_{20-25}$  concentrations. The sum of their effects was used to calculate the "real expected values" for the combinations.



**FIG. 1.** Graphs of  $\text{log}_{10}$  concentration versus effect were plotted for each substance: L-glutamic acid (a), Brilliant Blue (b), aspartame (c), and Quinoline Yellow (d). Error bars represent standard error of the mean ( $n = 4$ ). The concentration at which 50% reduction in neurite outgrowth from NB2a neuroblastoma cells in vitro was achieved was assessed for each additive ( $\text{IC}_{50}$ ), and concentrations producing lower ( $\text{IC}_{20-25}$ ), higher ( $\text{IC}_{70}$ ) or no inhibition of neurite outgrowth were also determined from the concentration-response curves, for use in experiments to assess possible combination effects.

Significant synergy was observed in the interaction between Brilliant Blue and L-glutamic acid (Fig. 2a), in various proportions (up to 0.1 nM Brilliant Blue and up to 1  $\mu\text{M}$  L-glutamic acid) of the  $\text{IC}_{20}$ ; the 50:50 mixture produced 46.1 (4.6)% neurite inhibition (mean (SEM),  $n = 4$ ), statistically significantly different ( $p < 0.005$ ) from the expected value of 15.8 (4.6)%. Figure 3 displays images of differentiating NB2a cells. A marked reduction in the average length of neurites was observed in cells treated with the 50:50 mixture of Brilliant Blue and L-glutamic acid (Fig. 3d), in comparison to those found in cells treated with the  $\text{IC}_{20}$  values of either Brilliant Blue (Fig. 3b) or L-glutamic acid (Fig. 3c) alone.

Synergy was also observed in the interaction between Quinoline Yellow and aspartame (Fig. 2b), in various proportions (up to 1  $\mu\text{M}$  of each) of the  $\text{IC}_{25}$ ; the 50:50 mixture produced 68.6 (3.7)% neurite inhibition, statistically significantly different ( $p < 0.0001$ ) from the expected value of 26.7 (3.2)%.

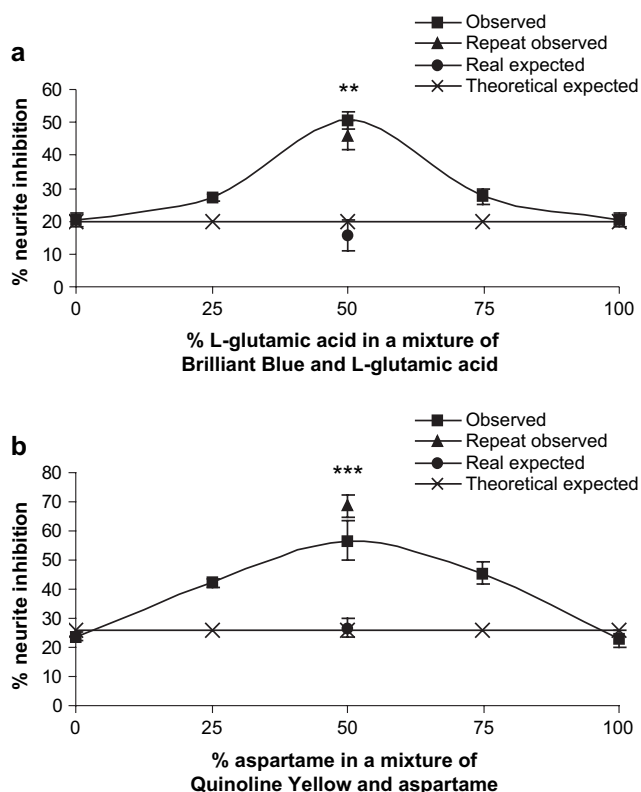
#### Assessment of Synergistic Neurotoxicity of Food Additive Combinations: Measurement of Interactions by "Dose Additivity"

Individual concentration-response curves for all four additives were used to calculate the individual concentrations required to produce 50% inhibition of neurite outgrowth (Fig. 1). Additivity isobolograms were constructed (Fig. 4).

The iso-effective concentrations of L-glutamic acid and Brilliant Blue were 48.7 (9.7)  $\mu\text{M}$  and 51.4 (21.2) nM re-

spectively. All mixtures tested generated data points below the line of additivity. For example, a combination of 10 (2.7)  $\mu\text{M}$  L-glutamic acid (mean (SEM),  $n = 4$ ) and 10 nM BB was required to produce an effect of 50% inhibition neurite outgrowth. Therefore the generated data point lies below the additivity line, producing a concave isobole (Fig. 4a). When incorporating this data into Equation 1, ( $c_A/C_A + c_B/C_B = 1$ ):  $10/48.7 + 10/51.4 = 0.4$ , the equation becomes  $c_A/C_A + c_B/C_B < 1$ . Brilliant Blue and L-glutamic acid thus interacted synergistically. The total concentration (SEM) of the mixture for 50% inhibition of neurite outgrowth was 10.01 (2.7)  $\mu\text{M}$ , whereas simple additivity predicts 39.2 (8.7)  $\mu\text{M}$ , as calculated using Equations 2 and 3. This difference was highly significant ( $p < 0.01$ ) as determined by the *t*-test on the logarithmic scale.

The iso-effective concentrations of aspartame and Quinoline Yellow were 153 (51.5)  $\mu\text{M}$  and 106 (31.8)  $\mu\text{M}$  respectively. Again all mixtures tested generated data points below the line of additivity. A combination of 8.06 (2.1)  $\mu\text{M}$  aspartame and 10  $\mu\text{M}$  QY was required to produce an effect of 50% inhibition of neurite outgrowth. Similarly, the generated data point lies below the additivity line (Fig. 4b), and when incorporated into Equation 1:  $8.06/153 + 10/106 = 0.1$ , the equation becomes  $c_A/C_A + c_B/C_B < 1$ ; thus these compounds also interacted synergistically. The total concentration (SEM) of the mixture for 50% inhibition of neurite outgrowth was 18.06 (2.1)  $\mu\text{M}$ , whereas simple additivity predicts 138.6 (48.6)  $\mu\text{M}$ . This difference was also highly significant ( $p < 0.01$ ).



**FIG. 2.** Results show the relationship between inhibition of neurite outgrowth from NB2a neuroblastoma cells and the percentage of each compound in a mixture. Error bars represent standard error of the mean ( $n = 4$ ). Both combinations of Brilliant Blue with L-glutamic acid (a), and Quinoline Yellow with aspartame (b) produced significantly more inhibition of neurite outgrowth than the individual compounds,  $**p < 0.005$  and  $***p < 0.0001$ , respectively.

#### Role of NMDA Receptors in Food Additive Induced Neurotoxicity

Preliminary experiments showed that exposure to CNS-1102 alone, at concentrations between 0.1–10  $\mu\text{M}$ , had no neurotoxic effect on NB2a cells. At concentrations above 100  $\mu\text{M}$ , complete neurite inhibition was observed (data not shown). The addition of CNS-1102 did not prevent food color-induced neurotoxicity (Figs. 5a, 5b); however, it did reduce neurotoxicity caused by L-glutamic acid and aspartame (Figs. 5c, 5d). L-glutamic acid alone produced 64 (7.9)% neurite inhibition (mean (SEM),  $n = 4$ ), compared to the 11.9 (3.6)% produced in the presence of 0.1  $\mu\text{M}$  CNS-1102 ( $p < 0.005$ ). Similarly, aspartame alone produced 64.8 (3.9)% neurite inhibition, while no neurite inhibition was observed in the presence of 0.1  $\mu\text{M}$  CNS-1102 ( $p < 0.005$ ). These results demonstrate that L-glutamic acid and aspartame-induced neurite inhibition was mediated by NMDA receptor activity.

#### Cytotoxic Effect of the Food Additives on NB2a

Viability was assessed by Trypan Blue dye exclusion, which gave a measure of cell mortality (Fig. 6). The effect additivity

model was used to analyze interactions. Combinations of Brilliant Blue with L-glutamic acid, and Quinoline Yellow with aspartame did not produce significantly more cytotoxicity than that expected from the sum of the individual additives. The observed 50:50 mixture of Brilliant Blue with L-glutamic acid produced 72.5 (5.5)% cell death (mean (SEM),  $n = 4$ ), which was not statistically significantly different from the expected value of 66.4 (2.2)% (Fig. 6a). The observed 50:50 mixture of Quinoline Yellow with aspartame produced 58.4 (3.1)% cell death, which again was not statistically significantly different from the expected value of 54.6 (3.6)% (Fig. 6b). The interactions were simply additive.

#### Additive Content in Foods and Theoretical Exposure

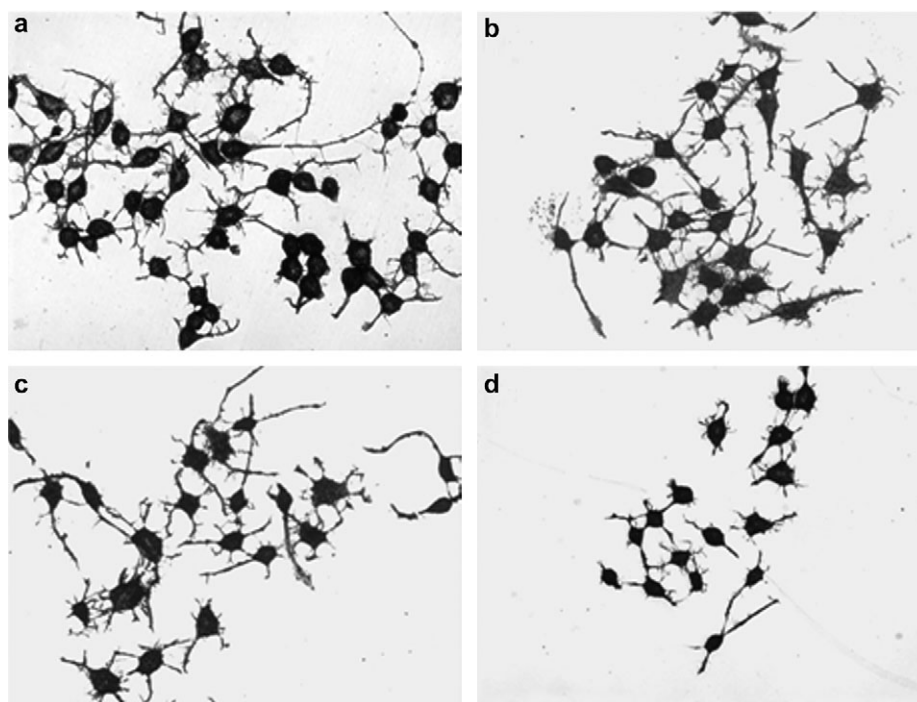
Five commercial products were analyzed for specific additive content. Results are shown in Table 1. Many of the products contained more than one additive. The potential plasma concentrations of additive reached following ingestion of certain products by a 10 kg child were found to be similar to concentrations required to cause neurite inhibition in the *in vitro* model of neurotoxicity (Table 2). In calculating these data, assumptions have been made in estimating exposure, i.e., calculations of absorption, distribution, and metabolism can be made from the available data, but are only an approximation of actual exposure.

## DISCUSSION

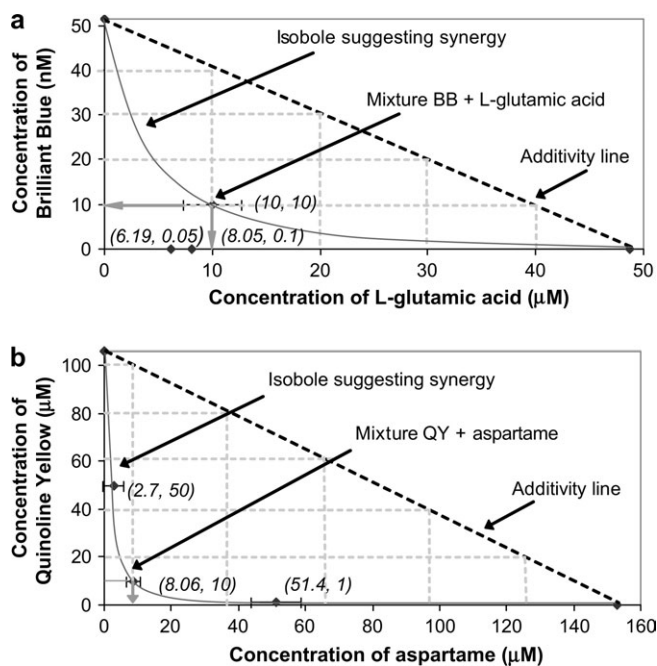
The association of food additives with hyperactivity is a popularly accepted notion. Feingold (1975) hypothesized that food dyes are pharmacologically active substances that induce or aggravate symptoms of hyperactivity in children. Subsequent studies have confirmed that food colors can induce clinical symptoms of hyperactivity (Bateman *et al.*, 2004; Boris and Mandel, 1994) and can also alter brain electrical activity in a subgroup of children with ADHD (Uhlig *et al.*, 1997).

Yet there is still no conclusive scientific evidence to indicate that any of the currently available food additives have any adverse effect on human development. The present study investigated the developmental neurotoxic effects of four common food additives. Two independent models were used to assess interactions in this study: “effect additivity” and “dose additivity.” Combinations acted synergistically in reducing the length of neurite outgrowth from differentiating mouse NB2a neuroblastoma cells. Quinoline Yellow and aspartame showed greater synergy than Brilliant Blue and L-glutamic acid; however, the results indicate that both combinations are potentially more toxic than might be predicted from the sum of their individual compounds.

The colors examined in this study are synthetic dyes that are certified as safe and are permitted for use as food additives in



**FIG. 3.** Images of Coomassie Blue-stained differentiating NB2a cells: in the absence of additive (a), or in the presence of 0.1nM BB to produce approximately 20% neurite inhibition (b), 1  $\mu$ M L-glutamic acid to produce approximately 20% neurite inhibition (c), and a mixture of Brilliant Blue and L-glutamic acid each at 50% of the above concentrations (d). Cells were viewed in a Zeiss Axiovert 35 M microscope on PH1, X20. The full field width of each image is 315  $\mu$ m.

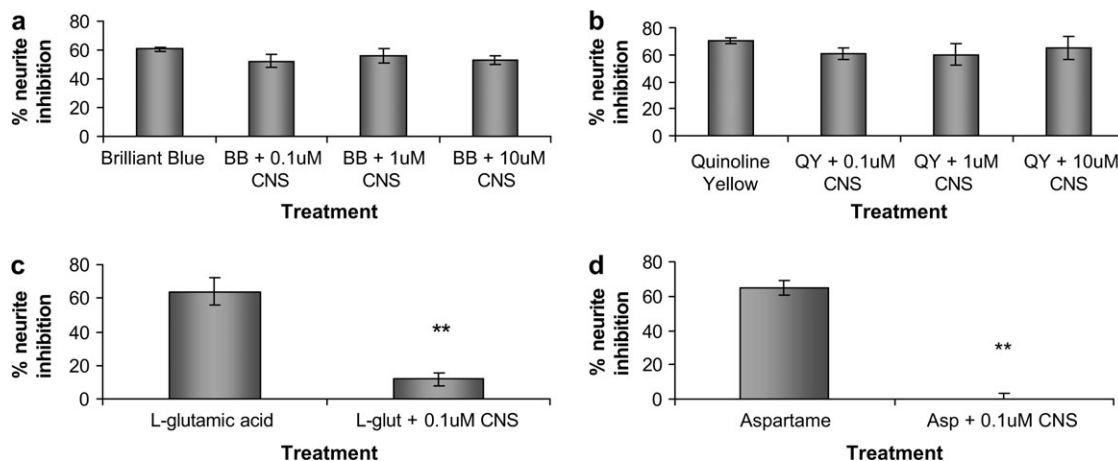


**FIG. 4.** Dose-additivity isobolograms for 50% neurite inhibition: mixtures of Brilliant Blue and L-glutamic acid (a), and mixtures of Quinoline Yellow and aspartame (b). Each isobologram experiment was performed four times independently of each other; data presented for each food additive combination are a mean of all four experiments. Horizontal error bars represent standard error of the mean ( $n = 4$ ). Both combinations of compounds were found to interact synergistically for the specified effect level.

the U.K. Brilliant Blue (E133) is banned in the majority of the EU countries and causes mitochondrial toxicity *in vitro* (Reyes *et al.*, 1996). The use of Quinoline Yellow (E104) in foods is banned in Australia, Norway, and the U.S., and genotoxic effects have been reported (Macioszek and Kononowicz, 2004). However, very little information about the neurotoxicity of food colors is available, and the mechanism by which they exert their toxic effect on nerve cells is not clear.

In contrast, the excitatory amino acids (EAA), L-glutamic acid, and aspartic acid are well established neurotoxins. Over three decades ago, it was discovered that L-glutamic acid destroys dendrites and cell bodies of neurons in the developing brain, thus causing brain lesions (Olney, 1969a). Oral and subcutaneous administration of L-glutamic acid to infant animals (rodents and primates) induces acute neuronal necrosis in several regions of the developing brain including the hypothalamus and the hippocampus (Kubo *et al.*, 1993; Olney *et al.*, 1972). As adults, treated animals show stunted skeletal development, obesity, and female sterility (Olney, 1969a). Retinal neuronal changes also occur in rats after prolonged administration of high L-glutamic acid diets (Ohguro *et al.*, 2002), whilst in adult humans, it elicits headache in susceptible individuals and is believed to be responsible for the “Chinese Restaurant Syndrome” (Schaumburg *et al.*, 1969), symptoms of which include chest pain, numbness, burning and facial pressure.

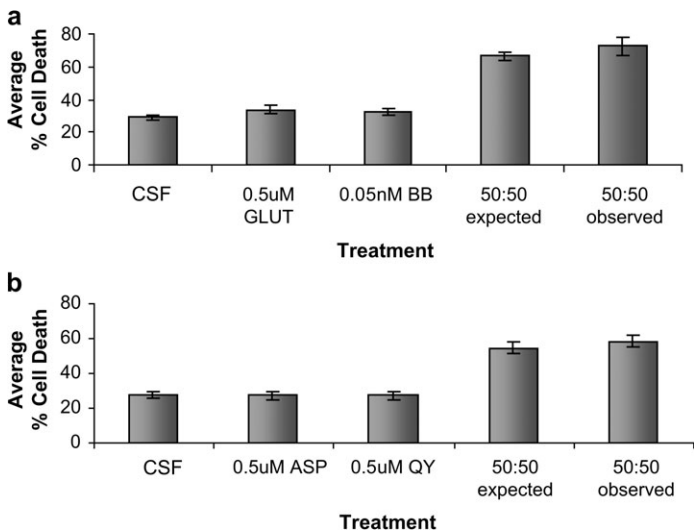
Similar hypothalamic lesions can be induced by aspartic acid, one of two of the constituent amino acids in the dipeptide



**FIG. 5.** Average percentage neurite inhibition: NB2a cells treated with IC<sub>70</sub> for Brilliant Blue (a), Quinoline Yellow (b), L-glutamic acid (c), and aspartame (d), in the absence (control) and presence of CNS-1102. Error bars represent standard error of the mean (*n* = 4). Addition of 0.1 μM CNS-1102 significantly reduced the neurotoxic effects of L-glutamic acid (\*\**p* < 0.005) and aspartame (\*\**p* < 0.005); however the antagonist at up to 10 μM did not prevent food color-induced neurotoxicity.

sweetener aspartame (Olney and Ho, 1970). Following ingestion, aspartame is rapidly hydrolyzed to release three biologically active chemicals: aspartic acid, phenylalanine, and methanol, which are absorbed into the portal blood (Burgert *et al.*, 1991; Ranney and Oppermann, 1979). It has been commonly used in diet drinks and sugar-free foods throughout the world for over 20 years, despite reports of panic attacks, seizures, and headaches with its use (Blumenthal and Vance, 1997; Drake, 1986; Walton, 1986). Recently, chronic exposure of aspartame was found to affect memory in rats (Christian *et al.*, 2004).

Excitotoxins destroy central neurons by excessive stimulation of postsynaptic excitatory membrane receptors (Rothman and Olney, 1995), whereas the under-stimulation of such receptors during the developmental period triggers apoptosis (Ikonomidou, 1999). Thus, excitotoxic and apoptotic neurodegeneration are two distinct cell death processes that are readily distinguishable ultrastructurally (Ishimaru *et al.*, 1999). It is well established that an excitotoxic mechanism plays a role in many neurologic disorders, from acute insults such as stroke and head trauma to chronic neurodegenerative states such as Huntington's disease and the acquired immunodeficiency syndrome (AIDS) dementia complex (Choi, 1988; Lipton and Rosenburg, 1994; Meldrum and Garthwaite, 1990). The overstimulation of such receptors leads to the opening of voltage-dependant calcium channels, initiating a cascade of events involving the activation of protein kinases, phospholipases, proteases, nitric oxide synthase (NOS), generation of free radicals and mitochondrial damage (Beal, 1992). The NMDA receptor plays a prominent role because of its high permeability to Ca<sup>2+</sup>; however other EAA receptor subtypes also



**FIG. 6.** Mean percentage cell death determined by Trypan Blue dye exclusion for food additive combinations: Brilliant Blue with L-glutamic acid (a) and Quinoline Yellow with aspartame (b). Error bars represent standard error of the mean (*n* = 4). Both combinations had a straightforward additive effect on cytotoxicity.

**TABLE 1**  
Analysis of Specific Food Additive Content in Foodstuff

Product	Typical portion size	Additive	Additive content
Sugar-coated sweets	42.5 g	Quinoline Yellow	25 mg/kg
Sugar-coated sweets	42.5 g	Brilliant Blue	9 mg/kg
Fruit-flavored jelly beans	45 g	Quinoline Yellow	92 mg/kg
Fruit-flavored jelly beans	45 g	Brilliant Blue	6 mg/kg
Fruit juice drink	250 ml	Aspartame	340 mg/kg
Baked corn snacks 1	25 g	Aspartame	207 mg/kg
Baked corn snacks 1	25 g	L-glutamic acid	2.53%
Baked corn snacks 2	25 g	L-glutamic acid	2.71%

TABLE 2  
Theoretical Exposure to Additives Based on Analysis of Food Additive Content in Foodstuff and Percentage Absorption from the Gut

Product	Additive	Additive content	Potential plasma concentration	% Neurite inhibition
2 × Packet of sugar-coated sweets with a chocolate center	Brilliant Blue	9 mg/kg	10.1 nM	43.7
1 × Packet of baked corn snacks	L-glutamic acid	2.53%	0.37 mM	64.3
1 × Packet of fruit-flavored jelly beans	Quinoline Yellow	92 mg/kg	50.28 nM	11.2
2 × Carton of fruit juice drink	Aspartame	340 mg/kg	58 μM	45.3

*Note.* Potential plasma levels are those that might be reached following ingestion of products by a 10 kg child. For assumptions used in the calculation, see text.

contribute to these processes. Selective non-competitive NMDA antagonists such as MK-801 markedly protect CNS neurons against direct excitotoxic effects; this has been demonstrated in primary cultures of hippocampal neurons following L-glutamic acid exposure (Michaels and Rothman, 1990). Our data are consistent with a role for excitotoxicity in the mechanism of injury caused by some flavor-enhancing food additives. CNS-1102 (a NMDA receptor antagonist) protected against both L-glutamic acid and aspartame-induced neurite inhibition, whilst the results demonstrated that food color-induced neurotoxicity was not mediated by NMDA receptor activation.

When assessing cell death mechanisms of food additive combinations, we found that both combinations studied had a straightforward additive effect on cell viability, as measured by Trypan Blue dye exclusion. The mechanisms of synergistic neurotoxicity are therefore unrelated to effects on viability.

The list of non-nutritional additives in foods is extensive, and it is virtually impossible to hold a single chemical responsible for a particular dysfunction. For many of the commercial products analyzed, more than one additive was detected. Children's sweets were found to contain both Brilliant Blue and Quinoline Yellow, whilst corn snacks were found to contain both aspartame and L-glutamic acid. Humans are not only exposed to such simple mixtures, but also to complex mixtures of chemicals rather than to individual chemicals, yet they continue to be tested for toxicity in isolation from each other. Also present in the environment are numerous potentially neurotoxic compounds such as pesticides that get into foods somewhere along the chain from farm to plate. It has been estimated that we have in our bodies between 300 and 500 chemicals that did not exist 50 years ago (Howard, 1997). Thus mixture studies are important to elucidate whether these interactions or chronic exposure to such mixtures would cause deleterious effects to a developing child. Very few long-term experiments have been attempted, and cumulative toxic effects have hardly been explored at all.

Despite being a major factor relevant to clinical settings, combination pharmacology is a topic that has not received much attention. It is essential that such investigations are carried out by reliable experimental procedures and appropriate

statistical methods; however there is widespread disagreement over terminology, definitions, and models for the analysis of interactions (Berenbaum, 1989; Kortenkamp and Altenburger, 1998). Several methods for calculating the expected combination effect of two or more compounds are currently in use, the majority of which can be associated with two popular basic concepts known as effect additivity (Bliss, 1939) and dose additivity (Loewe, 1953). Effect additivity focuses on measuring the effects of mixtures at only one specified concentration for each compound, thus lacking the information on concentration-response relationships. Dose additivity is an equally valid procedure for analyzing interactions between agents irrespective of their mechanisms of action, and aims to establish the required concentrations of individual compounds within a combination that produces a specified level of effect. However, this method requires tedious testing with a variety of concentrations for the determination of each data point on the isobologram, where a vast amount of information is eventually lost. Furthermore, isobolographic analysis requires independent statistical analysis (Tallarida, 1992), which can be extremely complicated. There is no generally accepted agreement as to which of the two concepts is more appropriate; therefore we have attempted to carry out this study using both models to confirm our findings. Similar conclusions could be drawn from both methods.

During the developmental period of synaptogenesis (brain growth spurt period), neurons are very sensitive to specific disturbances in their synaptic environment (Olney, 2002). In humans, this period extends from the sixth month of gestation to several years after birth, thus children are considerably more vulnerable to harm from toxic chemicals than adults. Since they are at a crucial stage of development, exposure to toxic chemicals may directly or indirectly attack their undeveloped nervous, immune, and endocrine systems. Dysfunction in any of these systems may lead to deleterious health effects. Cell proliferation, migration, differentiation, and synapse formation progress in a tightly programmed and orderly fashion. Interference with any stage of this cascade of events may alter normal progression of subsequent stages and short-term disruptions may have long-term effects later in life. Neurotoxicants may interfere with brain development and subsequent



function at exposure levels that have minimal or no effect on the adult brain.

The *in vitro* cell line may of course be more susceptible to toxicity than an *in vivo* model. Specifically, the *in vitro* neurotoxicity assay has no representation of the blood-brain barrier (BBB); however this is not complete in the developing human brain until around six months after birth (Adinolfi, 1985). Furthermore, some regions of the brain are not protected by a BBB at any time in life (Brightman and Broadwell, 1976); thus they remain in contact with any potentially neurotoxic substances circulating in the blood. Such regions are known as the circumventricular organs (CVOs), which include the portal system of the hypothalamus. The CVOs make up a minor proportion of the brain but are functionally very important regions.

For the measurement of potential body concentrations following ingestion, a number of assumptions have been made in our calculations. Absorption and distribution of additives need to be taken into account when relating *in vitro* data to *in vivo* effects, however there is little information about the absorption from the gut in infants or their distribution in the brain. In all cases, the potential whole body volume exposure to individually assessed additives lies within the range that we found to reduce neurite outgrowth by approximately 10–20% for Quinoline Yellow, 40–50% for Brilliant Blue and aspartame, and 50–60% for L-glutamic acid. Furthermore, neurite outgrowth would be reduced significantly more if the compounds were assessed in combination.

In conclusion, we present evidence that specific combinations of common food additives show synergistic effects to inhibit neuronal cell differentiation *in vitro*, using both the effect additivity and dose additivity models of assessing interactions. The immature nervous system may be vulnerable to such toxic insults since this marker of neurotoxicity was found at concentrations of additives theoretically achievable in plasma by ingestion of a snack and/or drink typically consumed by children. Mechanisms of synergistic toxicity have yet to be determined, and the implications of these data on developmental disorders remain to be investigated.

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