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Investigating the Cytotoxic and Calcium Signalling Impacts of Yellow 5 and Methyl Yellow Dyes in Mouse Nerve Cells

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Abstract **-** The widespread use of artificial food dyes in consumables and cosmetics has raised critical concerns regarding their potential adverse effects on human health. This study evaluates the neuro-cytotoxic and calcium signaling impacts of Tartrazine (Yellow 5), a commonly used dye, and Methyl Yellow, a dye banned in the 1930s due to its toxicity. Using cultured mouse hippocampal nerve cells as a model, we assessed cell viability through Ethidium Homodimer III/Dead Red staining and fluorescence imaging after exposure to varying concentrations of Yellow 5 and Methyl Yellow. Calcium imaging with Fluo-4 revealed disruptions in calcium buffering and signaling pathways, which are critical for cellular homeostasis. These findings underscore the potential of artificial food dyes to compromise cellular function and amplify environmental stressors. In light of California's 2024 legislation banning six artificial food dyes, including Yellow 5, this study contributes new insights into food additive safety and emphasizes the necessity of rigorous oversight to safeguard consumer health.

Keywords**:** Cytotoxicity, Calcium Signaling, Yellow 5, Methyl Yellow, Hippocampal Cells, Cortical Cells, Dead Red Staining, Fluo-4, Oxidative Stress, Food Safety Regulations

1. Introduction

Artificial food dyes have been used extensively for decades to enhance the visual appeal of processed foods, beverages, and cosmetics. Tartrazine (Yellow 5, E102) dominates the synthetic colorant market, with annual global production exceeding 10,000 metric tons, primarily consumed in carbonated drinks (30%), confectionery (25%), and snack foods (20%) [1]. In the U.S., the FDA certifies over 3.2 million pounds of Yellow 5 annually, with children aged 4-12 estimated to consume 1.1-2.3 mg/kg body weight daily [2]. Despite its prevalence, studies link Tartrazine to neurotoxicity, oxidative stress, and behavioral disturbances [3-5], with recent evidence showing it induces structural and functional aberrations in vivo [6]. The structurally similar Methyl Yellow (Butter Yellow) serves as a historical comparator, having been banned in 1938 after being linked to hepatic tumors in rats, following its previous use in 500 tons/year of margarine and dairy products [7], [8]. Residual environmental contamination persists, with 4-12 μg/L detected in groundwater near former production sites [9].

This study investigates the neuro-cytotoxic effects and calcium signaling alterations induced by Yellow 5 and Methyl Yellow using cultured mouse hippocampal neurons. Calcium imaging with Fluo-4 revealed significant disruptions in calcium homeostasis upon addition of Yellow 5 and Methyl Yellow. Additionally, we subjected cells to laser induced shockwave(SW) to determine the effects of Yellow 5 on injury responses. Laser induced shockwave allows us to subject neurons to injury similar to that caused by a blast induced traumatic brain injury [10].

The neurotoxic potential of these food dyes gains additional urgency from recent findings showing synergistic interactions between commonly used food additives in developmental neurotoxicity tests [11], along with regulatory developments. California's 2024 prohibition of six artificial dyes (including Yellow 5) from school meals [12] and the EU's mandatory warning labels [13] contrast sharply with the FDA's current 100 mg/kg permissible limit [14]. This regulatory disparity reflects an expanding body of evidence linking synthetic dye exposure to neurobehavioral impairments, including a 32% higher ADHD prevalence (95% CI: 1.15-1.52) in epidemiological studies [15, 16]. Mechanistic research has further demonstrated these additives' capacity to disrupt neurotransmitter function [17, 18] and induce cognitive deficits in developing brains [5, 19].

This study provides a comprehensive risk assessment of artificial food dyes by bridging historical toxicity data (Methyl Yellow) with contemporary neurobiological evidence (Yellow 5). Our multidisciplinary approach - combining cytotoxicity assays, calcium imaging, and shockwave (SW) stress evaluations - advances understanding of their cellular mechanisms while reinforcing the need for evidence-based food safety policies. The findings particularly highlight the vulnerability of pediatric populations and underscore the importance of aligning regulatory standards with current scientific evidence.

2. Materials and Methods

2.1 Cell Culture and Preparation

Primary hippocampal neurons were dissociated from postnatal day 0 (P0) mouse pups. Cells were plated on poly-D-lysine-coated glass-bottom dishes and cultured in neuronal maintenance media at 37°C, 5% CO_2, and 10% humidity.

2.2 Calcium Imaging with Food Dye Exposure

At day in vitro 4 (DIV4), neurons were incubated with Fluo-4 AM (4.5 μg/mL) for 35 minutes, washed, and then maintained in medium supplemented with 1 μg/mL Ethidium Homodimer III (Dead Red) for dead cell detection. Yellow 5 and Methyl Yellow were applied at various concentrations (50 μM and 100 μM). Time-lapse fluorescence imaging captured calcium dynamics over 20 minutes.

2.3 Laser-Induced Shockwave (LIS) Injury

To simulate mechanical injury, neurons were subjected to laser-induced shockwave (LIS) using a femtosecond laser system. Calcium responses were recorded before and after LIS, and ImageJ software was used to quantify the radius of cell death and detachment.

2.4 Data Analysis

Fluorescence intensity changes ($\Delta F/F_0$) were quantified to evaluate calcium signaling alterations. Percent changes, area under the curve (AUC) analyses, and cell death radii were calculated for each condition in in-house Matlab APP. Comparisons were made between control, Yellow 5-treated, and Methyl Yellow-treated groups.

3. Results and Discussions

3.1 Effects of Yellow 5 and Methyl Yellow on Calcium Signaling

Upon addition of food dyes, significant disruptions in intracellular calcium dynamics were observed in mouse hippocampal neurons. Control treatments (water or DMSO) induced only a modest 32% increase in fluorescence within 2.5 minutes. In contrast, 50 μM Yellow 5 treatment triggered a 90% rise, while 100 μM Methyl Yellow(MeY) exposure led to a 119% increase (Figure 1A). Curiously, 100 μM Yellow 5 showed a lower rise 51% compared to 50 μM. For Yellow 5, comparisons were also made when it was dissolved in medium instead of water. Dissolution in medium was meant to ensure that the cells would not experience calcium alterations due to osmotic shock. The addition of medium alone led to a mean 23% increase in 16 out of 50 cells quantified compared to a 70% increase (in 44 of 50 quantified cells) when 50 μM Yellow 5 was added. Similar to previous results in Figure 1A, 100 μM Yellow 5 led to a smaller calcium increase of 34% than the 50 μM Yellow 5 concentration. The results suggest that the higher concentration of 100 μM Yellow 5 may be causing unwanted interactions with Fluo4-AM causing it to fluoresce less in the presence of calcium.

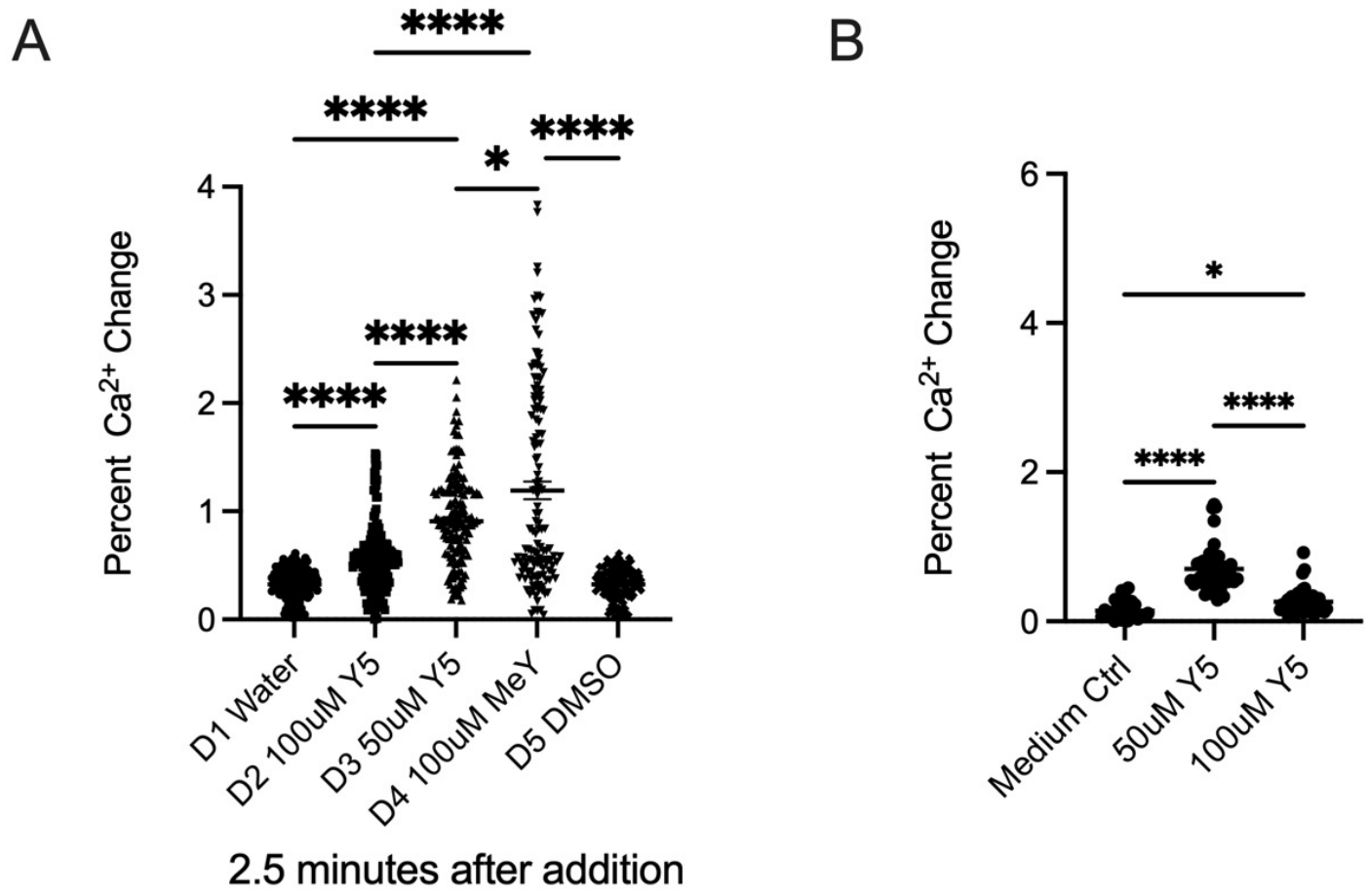

Figure 1. Calcium percent change 2.5 minutes after addition of dye solvents and food dyes. * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$

Calcium elevations due to the food dyes persisted throughout the 20-minute imaging period, indicating prolonged calcium dysregulation (Figure 2A). Area-under-the-curve (AUC) analysis confirmed that both 50 μM Yellow 5 and 100 μM Methyl Yellow produced substantially greater cumulative calcium displacements than controls, with Methyl Yellow exhibiting the most pronounced effect (Figure 2B). These results suggest that both dyes can disrupt calcium homeostasis, a key regulator of neuronal survival and signaling integrity. Interestingly, a slight reduction in calcium elevation was noted with 100 μM Yellow 5 compared to 50 μM, potentially due to dye aggregation at higher concentrations interfering with Fluo-4 AM fluorescence or cellular uptake.

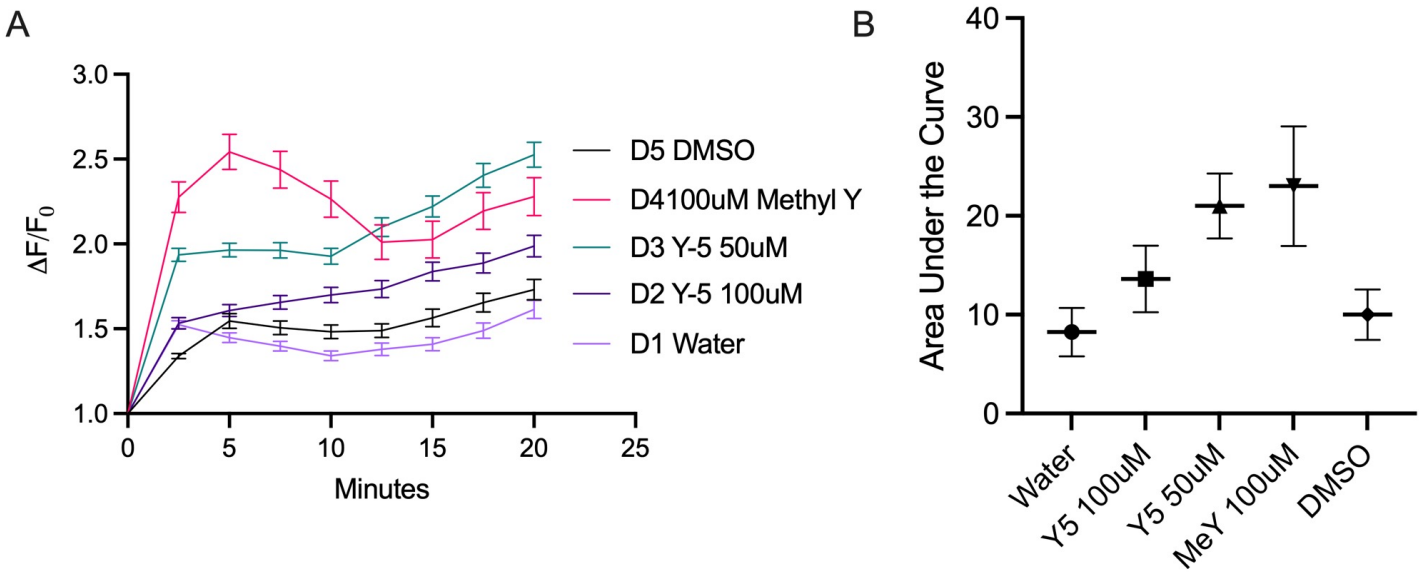

Figure 2. Calcium levels up to 20 minutes post dye addition(A). The area under each curve is plotted in (B). Standard error means are shown in error bars.

3.2 Impact of Yellow 5 on Neuronal Calcium Injury Responses Following Laser-Induced Shockwave

To investigate the potential exacerbating effects of Yellow 5 under mechanical injury conditions, laser-induced shockwave (LIS) was applied to neuronal cultures and the subsequent calcium response was studied. The percent change after shockwave was significantly larger than food dye alone (Figure 3A). The duration of the increase, measured by the full width half max(FWHM) of the spike, showed that at 50 μM Yellow 5 the elevation following shockwave was shorter in duration than shockwave in medium control. Results for area under the curve showed that shockwave leads to greater calcium displacements than dye additions alone. The area under the curve was smaller for cells shockwaved in 50 μM Yellow 5 than medium controls, consistent with FWHM results. These results indicate that the Yellow 5 is having an effect in the cells injury response or that the dye may be leading to buffering of the calcium. Additionally, the dye may be non specifically binding cellular components responsible for the injury response.

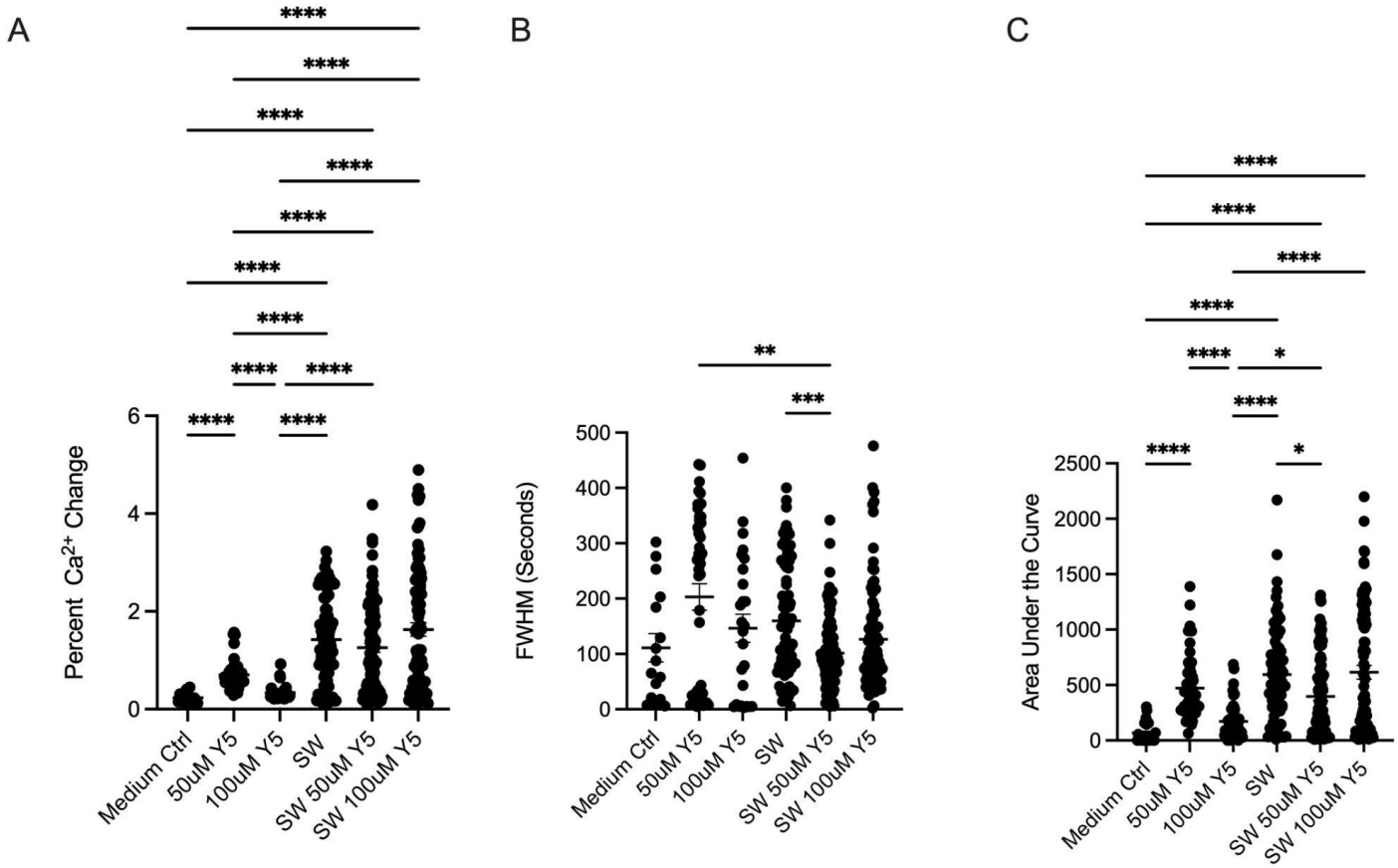

Figure 3. **Calcium changes for cells in control conditions(medium) or in Yellow 5 dissolved in medium and with or** without shockwave(SW). (A) Percent calcium changes are shown. (B) The duration of the calcium spike is quantified by calculating the full width half maximum(FWHM). (C) The area under the curve allows us to get an idea of the total calcium displacement. * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$

3.3 Impact of Yellow 5 on Neuronal Cell Death Following Laser-Induced Shockwave (SW)

Dead Red staining and brightfield imaging revealed that cells exposed to SW alone had a smaller mean radius of cell death and detachment (18.1 μm) compared to those exposed to SW combined with Yellow 5 (20.92 μm), suggesting increased vulnerability when Yellow 5 was present (Figure 4).

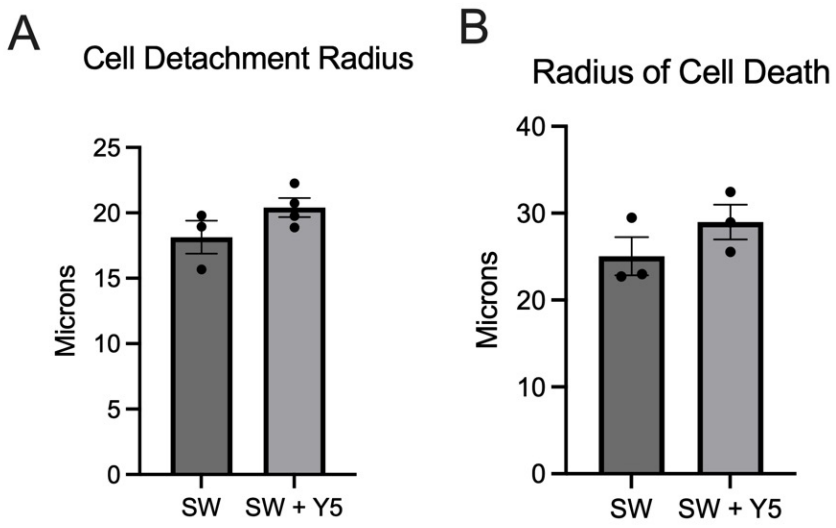

Figure 4. Radius of cell detachment and cell death after laser induced shockwave (SW). No statistical differences were found for the small sample size. However, the average may be higher in the presence of Yellow 5.

3.3 Summary of Key Findings

- **Disrupted Calcium Homeostasis**: Both Yellow 5 and Methyl Yellow induced persistent and exaggerated calcium elevations compared to controls, implicating severe intracellular signaling disturbances.
- **Increased Susceptibility to Mechanical Injury**: Neurons pre-treated with Yellow 5 exhibited larger radii of cell death and attenuated calcium responses post-SW, suggesting heightened fragility. Future studies with greater n-values will test this hypothesis.
- **Dose-Dependent Effects**: At higher dosages (e.g., 100 μM Yellow 5) lower calcium disruption was observed. These results suggest that at this concentration an interaction with the calcium indicator Fluo-4 AM may be occurring or the dye itself may be complexing with itself preventing uptake into the cell. Future studies will look at the calcium response in cells treated with a different indicator whose spectrum should not overlap with that Yellow 5. Additionally, a lower concentration of 25 μM Yellow 5 will be tested.

These results collectively demonstrate that exposure to synthetic food dyes compromises neuronal health by both altering baseline calcium signaling and reducing resilience to traumatic injury.

4. Conclusion

This study provides important preliminary evidence that synthetic food dyes, including Yellow 5 (Tartrazine) and Methyl Yellow, exert significant neurotoxic effects on mouse hippocampal neurons. Exposure to both dyes led to pronounced disruptions in intracellular calcium homeostasis, as demonstrated by sustained elevations in calcium fluorescence intensity and increased area-under-the-curve (AUC) measurements. These alterations suggest critical impairments in cellular signaling pathways vital for neuronal survival and function.

Moreover, neurons pre-exposed to Yellow 5 displayed heightened vulnerability to laser-induced shockwave (LIS) injury, as indicated by an expanded radius of cell death and a dampened calcium transient response following mechanical trauma. Although not yet statistically significant due to limited sample sizes, the observed trends strongly support the hypothesis that synthetic food dyes compromise neuronal resilience under oxidative and mechanical stress conditions.

Together, these findings highlight the potential risks posed by common artificial food dyes to neuronal health, particularly in contexts involving environmental or physiological stressors. They reinforce growing concerns that exposure to such additives may contribute to neurodevelopmental and neurodegenerative processes.

Future work will involve expanding sample sizes, incorporating additional functional assays (e.g., reactive oxygen species detection, mitochondrial function analysis), and exploring protective strategies to mitigate dye-induced neurotoxicity. Our preliminary data underscore the critical need for continued evaluation of food additive safety, particularly in vulnerable populations such as developing children.

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