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BAGUMA, Marius; KESSELS, Sofie; BITO, Virginie; BRONE, Bert; Triller, Antoine; Maynard, Stephanie; Legendre, Pascal; RIGO, Jean-Michel; Le Corronc, Herve & Chabwine, Joelle Nsimire (2024) New insight into the molecular etiopathogenesis of konzo: Cyanate could be a plausible neurotoxin contributing to konzo, contrary to thiocyanate. In: Neurotoxicology, 105 , p. 323 -333.

DOI: 10.1016/j.neuro.2024.11.004
Handle: http://hdl.handle.net/1942/45042

New insight into the molecular etiopathogenesis of konzo: Cyanate could be a plausible neurotoxin contributing to konzo, contrary to thiocyanate

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Abstract

Introduction: Chronic cassava-derived cyanide poisoning is associated with the appearance of konzo, a tropical spastic paraparesis due to selective upper motor neuron damage. Whether the disease is caused by a direct action of cyanide or its metabolites is still an open question. This preliminary study assessed the neurotoxic effects of thiocyanate (SCN) and cyanate (OCN), two cyanide metabolites hypothesized to be plausible toxic agents in konzo.

Methods: Cultured mouse neuroblastoma (Neuro-2A) and human neuroblastoma (SH-SY5Y) cell lines were incubated (24, 48, and 72 hours) in sodium OCN or sodium SCN in a disease-relevant concentration range. Cell viability, caspase (3, 8, and 9) activities, and reactive oxygen species (ROS) generation were evaluated using appropriate assay kits. Additionally, electrophysiological responses induced by OCN and SCN in primary spinal cord neurons (from Sprague Dawley rats) were assessed by whole-cell patch-clamp techniques.

Results: Both OCN and SCN were toxic in a dose-dependent way, even if SCN toxicity appeared at very high concentrations (30 mM, corresponding to more than 100-fold higher than normal plasmatic levels), contrary to OCN (0.3-3 mM). OCN was markedly more toxic in a poor culture medium (MEM; IC50 = 3.2 mM) compared to a glucose- and amino acid-rich medium (DMEM; IC50=7.6 mM). OCN treatment increased the ROS generation by 8.9 folds, as well as the Caspase-3, Caspase-8, and Caspase-9 activities by 3.2, 2.5, and 2.6 folds, respectively. Finally, OCN (and SCN to a lesser extent) induced depolarizing currents in primary spinal cord neurons, through an activation of ionotropic glutamate receptors.

Conclusion: Our results suggest OCN as the most plausible neurotoxic agent involved in konzo, while SCN toxicity could be questioned at such high concentrations. Also, they support apoptosis, oxidative stress, and excitotoxicity as probable mechanisms of OCN neurotoxicity.

Keywords: Cyanide poisoning; cyanate; thiocyanate; neurotoxicity; konzo; cell culture.

1. Introduction

Hydrogen cyanide is one of the most powerful poisons for human beings (Teles, 2002). Acute cyanide poisoning may occur in various situations, including, but not limited to: suicide attempt, smoking, industrial exposure, ingestion of drugs such as sodium nitroprusside, accidental inhalation of toxic smoke during structural fires, or ingestion of some foods such as cassava (*Manihot esculenta* Crantz), bitter almond or apricot kernels, and bamboo shoots (Aranguri-llerena and Siche, 2020; Coentrão and Moura, 2011).

However, once cyanide is released in the organism from ingested cyanide-containing products, detoxification occurs following various metabolic pathways. Among them, cyanide transformation into thiocyanate (SCN), via trans-sulphuration reactions catalyzed by the rhodanese and beta-mercaptopyruvate sulfur transferase, constitutes the most prominent mechanism (~85% of ingested cyanide) (Nambisan, 2011; Oluwole, 2015; Tor-Agbidye et al., 1999). Other minor pathways may be engaged, especially in nutritionally compromised individuals, such as cyanide oxidation into cyanate (OCN) (Kassa et al., 2011; Tor-Agbidye et al., 1999). These detoxification mechanisms rescue from cyanide ingestion below lethal doses (Egekeze and Oehme, 1980).

The cyanide toxicity of cassava (the most produced staple crop caloric source in Africa and the fourth most important food crop worldwide (Food and Agriculture Organization of the United Nations, 2017; Sayre et al., 2011)), is due to cyanogenic glucosides (mainly linamarin (Montagnac et al., 2009)) contained in its roots and leaves (Njankouo et al., 2019). Therefore, high cyanogen-containing cassava roots need prior processing to ensure safe consumption (Baguma et al., 2022; Nzwalo and Cliff, 2011). Efficient processing techniques allow linamarin conversion into the toxic hydrogen cyanide (HCN) (Njankouo et al., 2019), which is thereafter eliminated from cassava roots by evaporation (Oluwole, 2015; Tshala-katumbay and Spencer, 2007) prior to consumption.

In contrast, ingestion of inadequately processed cassava may result in potential hazards through acute cyanide poisoning (Zacarias et al., 2017). Furthermore, chronic consumption of insufficiently processed cassava foods has been associated with some neurological diseases, namely tropical ataxic neuropathy and konzo (Adamolekun, 2011). Konzo is characterized by a symmetrical, non-progressive, and irreversible spastic paraparesis of sudden onset, and is due to a selective upper motoneuron damage (World Health Organization, 1996). The appearance of konzo is systematically associated with the presence of dietary cassava-derived cyanide exposure and deficiency in sulfur amino acids (SAA) in a context of malnutrition due to severe food deprivation (Baguma et al., 2021b; Tylleskar et al., 1991). Despite the constant epidemiological link observed between the occurrence

of konzo and dietary cyanide poisoning, neither the specific neurotoxic compound nor the disease pathogenic mechanisms have been elucidated (Baguma et al., 2021b; Bramble et al., 2021).

Whether konzo is caused by a direct effect of HCN or by its precursors (cyanogenic glucosides) or metabolites, remains an open question. Available observational and experimental data failed to establish a causal relationship between konzo and cyanide or any of the previously hypothesized metabolites (Baguma et al., 2021b). However, owing to their documented neurotoxic actions, SCN and OCN appear to be the most plausible candidates among cyanide metabolites, especially in populations undergoing malnutrition like in konzo-affected areas(Baguma et al., 2021b; Spencer, 1999). This experimental study aimed therefore at evaluating the neuronal toxicity of SCN and OCN and investigating mechanisms sustaining their neurotoxicity, with a possible protecting role of SAA. This strategy should allow a better understanding of pathogenic mechanisms underlying cassava-derived dietary cyanide poisoning contribution to konzo and the potential advantageous function of SAA from a preventive and/or therapeutic perspective.

2. Materials and Methods

2.1. Experimental concept and design, ethical considerations

The experiments primarily evaluated cell viability of primary cultured spinal cord neurons and two neuronal cell lines submitted to SCN and to OCN exposure. Furthermore, neuronal survival was comparatively studied in glucose- and SAA-enriched (Dulbecco's Modified Eagle's Medium (DMEM) - High Glucose) culture medium, referred to as DMEM in the remainder of the manuscript and in glucose- and SAA-poor environment (Minimum Essential Media (MEM)) to test the protective role of SAA.

In order to further enlighten involved pathogenic mechanisms, the caspase and oxidative stress pathways were targeted using standard biochemical methods on the same neuronal species. In addition, spinal cord primary cultured neurons were used for electrophysiological experiments to investigate the implication of excitotoxic mechanisms, including the type of glutamatergic receptors involved. Finally, immunocytochemistry experiments were used to compare the effects of SCN, OCN, and glutamate on the neuronal fragmentation process.

Unless otherwise specified, each experimental condition was conducted in quadruplets and data were generated from three independent experiments. Untreated cells were submitted to similar conditions as OCN- or SCN-treated cells, except exposure to the toxic compounds.

All experiments involving animals (primary cultures) were performed in accordance with the regulations of the French Ministry of Agriculture and approved by the "Direction Départementale des Services Vétérinaires de Paris" (Ecole Normale Supérieure, Animalerie des Rongeurs, license: B 75-05-20). All efforts were made to minimize the suffering of animals used and to reduce their number.

2.2. Cell viability experiments

These experiments were conducted in Mouse Neuro-2A neuroblastoma (ATCC CCL131) and human SH-SY5Y neuroblastoma (ATCC CRL2266) cell lines, which have been extensively used to investigate neurotoxic properties and mechanisms of several compounds (LePage et al., 2005) or as *in vitro* models of neurodegenerative diseases (Xicoy et al., 2017).

Cell viability upon OCN and SCN application was assessed using the 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (Merck Life Science BV, Overijse, Belgium), a colorimetric method measuring metabolic activity. This assay is based on the enzymatic reduction of MTT (a pale-yellow water-soluble tetrazolium salt) into formazan (water-insoluble purple crystals) retrieved in the mitochondria of viable cells (Lim et al., 2015) and catalyzed by mitochondrial succinate dehydrogenase. Since this transformation exclusively occurs in metabolically active cells , the amount of generated formazan reflects the number of viable cells (Wachsmann and Lamprecht, 2012). Experiments were performed in 96-well plates (Thermo Fisher Scientific, Merelbeke, Belgium), after 24h, 48h, or 72h incubation in various treatment conditions.

2.2.1. Neuron-like cell lines culture conditions and treatments

Before the experiments, Neuro-2A and SH-SY5Y cells were grown in clear sterile T75 flasks (Thermo Fisher Scientific, Merelbeke, Belgium) in DMEM (Merck Life Science UK Limited, Dorset, United Kingdom) supplemented with 10 % fetal bovine serum (Biowest®, South America). For SH-SY5Y cells, the culture medium was additionally supplemented with L-glutamine (2 mM; Merck Life Science BV, Overijse, Belgium) and penicillin-streptomycin (100 U/100 µg/ml; Merck Life Science BV, Overijse, Belgium). Cell cultures were maintained in an incubator in a humidified environment (5% CO2 at 37 °C). Cells were splited and culture medium changed every 3-4 days. All experiments were conducted on cells between passages 14-21 for Neuro-2A and 5-9 for SH-SY5Y cells. The sub-culturing was performed every 3-4 days, after reaching 75-90% of confluency.

Cells were seeded at a density of $2x10^4$ cells in 100 µl of the above-mentioned standard medium, in clear sterile 96 well-plates. The plates were incubated for 24 hours to allow cells to attach to the plate

bottom. Thereafter, the standard culture medium was carefully (and as completely as possible) removed from the wells using a multichannel pipet and replaced by the experimental solution (100 µl/well). The latter contained OCN or SCN in their commercial forms (respectively NaOCN (Alfa Aesar, Kandel, Germany) or NaSCN (Merck Life Science BV, Overijse, Belgium)) at increasing concentrations (0.03, 0.1, 0.3, 1, 3, 10, and 30 mM) and diluted either in fresh MEM (Gibco®, ThermoFisher Scientific, Merelbeke, Belgium) or in fresh DMEM (see below). All treatment solutions were freshly prepared within an hour before each experiment () from a 200 mM stock-solution kept at -20 °C for less than 30 days. Control experiments were performed using only fresh MEM or DMEM (*i.e.*, without OCN or SCN). All conditions were applied in quadruplicate. The treated cells were finally incubated for 24h, 48h, or 72h.

2.2.2. MTT Assay

After the incubation period, 10 µl of MTT diluted in phosphate-buffered saline (Lonza BioWhittaker®, Walkersville, MD, United States), was added to each well, and the latter kept in the dark for 3 hours at 37 °C to allow reduction of the MTT into formazan. The supernatant was then removed, and the formazan crystals dissolved in 100 µL of dimethyl sulfoxide (DMSO; VWR International BVBA, Leuven, Belgium). The obtained solution was mixed by shaking at 150 cycles/min using the Heidolph rotamax 120 orbital shaker (Heidolph Instruments GmbH & Co., Schwabach, Germany) at room temperature in the dark for 45 minutes. The optical density (OD) of each well was measured at 570 nm, using the iMark™ Microplate Absorbance Reader (Bio-Rad Laboratories GmbH, Feldkirchen, Germany). The final ODs were obtained after subtracting the mean OD of blank wells, *i.e.* wells not containing cells. The cell viability was expressed as percentages of surviving cells in treated sample relative to the control (untreated) sample, using the formula:

$$Cell\ viability\ (\%) = \frac{Mean\ ODs\ of\ treated\ sample}{Mean\ ODs\ of\ control\ sample}\ x\ 100$$

2.3. Assessment of mechanisms underlying OCN neurotoxicity in cell lines

2.3.1. Impact of enriched environment

The OCN toxicity on Neuro-2A and SH-SY5Y cells was measured after incubation in DMEM, an environment enriched with (4.5 times more) glucose, amino-acids (e.g. 2 times more SAA: L-methionine and L-cystine) and vitamins; in comparison with the standard incubation in MEM used

for the rest of experiments. Increasing OCN concentrations and incubation times were used, and cell viability measured using the MTT assay, as specified above.

2.3.2. Measurement of caspase activity

In order to assess the involvement of apoptosis in the OCN-induced toxicity, caspase-3, 8, and 9 activities were measured using their respective Multiplex Activity Fluorometric Assay Kits (ab219915; Abcam, Cambridge, United Kingdom) according to the manufacturer's protocol. Briefly, Neuro-2A cells were seeded in a sterile black 96-well plate with clear bottom (Ibidi GmbH, Gräfelfing, Germany) at a density of $2x10^4$ cells/well in standard medium and incubated for 24h for cell attachment. Then cells were treated with freshly prepared a 0.3 mM OCN solution diluted in a serum-free MEM. After 24h incubation in the experimental conditions, 100 µL of freshly prepared caspase assay loading solution was added to each well, without removing the treatment solution or the culture medium. The well was left for 1 hour in the dark and thereafter, the fluorescence intensity (measured in relative fluorescence units (RFU)) was measured at specific excitation/emission (Ex/Em) wavelengths corresponding to each caspase (respectively 540/612 nm, 490/520 nm 380/460 nm) using a Fluostar Optima microplate reader (BMG Labtech, Ortenberg, Germany). After subtraction of the blank readings from all measurements, the fluorescence activity of each group was normalized to the values of controls (untreated cells), using the formula:

$$Caspase\ activity = \frac{Mean\ RFU\ of\ treated\ \ cells}{Mean\ RFU\ of\ control\ cells}$$

Here, all conditions were applied in duplicates, while data were generated, as elsewhere, from three independent experiments.

2.3.3. Assessment of oxidative stress

To investigate the potential prooxidant effect of OCN, Neuro-2A cells were prepared as described in section 2.3.2 and treated with OCN 0.3 mM for 24 hours. Then the generated cellular reactive oxygen species (ROS) were measured using the fluorometric DCFDA/H2DCFDA Cellular ROS Assay Kit (ab113851; Abcam, Cambridge, United Kingdom) following the manufacturer's protocol. Fluorescence intensity was measured at Ex/Em = 485/520 nm using a Fluostar Optima microplate reader (BMG Labtech, Ortenberg, Germany). The blank readings were subtracted from all measurements, then the fluorescence activity of each group was normalized to the values of controls, using the formula:

$$Relative\ generated\ cellular\ ROS\ quantity = \frac{Mean\ RFU\ of\ treated\ cells}{Mean\ RFU\ of\ control\ cells}$$

Here also, all conditions were applied in duplicates, but data were generated from two independent experiments.

2.4. Electrophysiological experiments

2.4.1. Primary spinal cord neuronal culture preparation

Primary cultured mixed spinal cord neurons were prepared from C57BL/6J *Glrb*$^{Eos/Eos}$ mice embryos 13 days (E13) (Maynard et al., 2021). The spinal cord tissue was dissociated in papain (20 U/ml; Serolab) and DNase I (final concentration 0.1 mg/ml; Life Technologies) for 10 minutes at 37 °C, and centrifuged at 1000 rpm for 8 min. Neurons were plated on poly-DL-ornithine-coated (80 µg/ml; Sigma) 18 mm glass coverslips (thickness 0.16 mm, No. 1.5; VWR) at a density of 2.5 x 10^5 cells/cm^2 in Neurobasal medium (Life Technologies), supplemented with B-27 (Life Technologies), 2 mM glutamine (Life Technologies), and antibiotics (5 U/ml penicillin and 5 µg/ml streptomycin; Life Technologies). Neurons were kept at 37 °C and 5% CO_2. Medium was replenished every 4 days by replacing half of the volume with BrainPhys (StemCell Technologies) supplemented with SM1 (StemCell Technologies) and antibiotics. Primary cultured spinal cord neurons were utilized for electrophysiological recordings after 2 weeks in culture.

2.4.2 Patch-clamp recordings

Coverslips containing dissociated neurons were mounted on the stage of a microscope and continuously perfused (2 ml/min) at room temperature (20 - 24 °C) with artificial cerebrospinal fluid (ACSF). The ACSF was composed of (in mM) 130 NaCl, 4 KCl, 11 glucose, 0.5 $CaCl_2$, and 3 $MgCl_2$, 10 HEPES (pH was adjusted to 7.4 with NaOH, Osmolarity 310 mOsm/kg H_2O). Patch pipettes were made from borosilicate thick capillary glass and had of resistance of 2 – 3 3MΩ when filled with an intracellular solution containing (mM) 10 EGTA, 10 HEPES, 130 CsCl, 6 $MgCl_2$, 1 QX314-Cl, 4 Na_2ATP (pH adjusted to 7.2 with CsOH, osmolarity 290 mOsm/kg H_2O). The intracellular solution was set to obtain an equilibrium potential for chloride close to 0 mV, which was then equal to the equilibrium potential for cationic ions. In voltage clamp the membrane potential was held at Vh = -60 mV. ACSF and drugs were applied using 0.5-mm-diameter quartz tubing positioned 100 µm away from the recording area under direct visual control and allowing fast solution applications. All compounds were purchased from Sigma Chemicals except QX314 (Alomone Labs), tetrodotoxin

(TTX) (Latoxan), NaSCN (Merck) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and d-2-amino-5-phosphonopentanoic acid (d-AP5) (Tocris Bioscience).

Electrophysiological responses to SCN and OCN were measured using increasing concentrations of 1 mM, 3 mM, and 10 mM, based on the results of the preliminary experiments in cell line cultures. 30 µM Glutamate (Glu) was used as a positive control. To specifically measure specific glutamatergic receptor-mediated currents, CNQX at 20 µM and d-AP5 at 100 µM were used, whereas the voltage-gated sodium channels blocker TTX was applied at 0.5 µM in order to assess the involvement of glutamatergic transmission. The duration of the application was 5 seconds for glutamate and 10 seconds for OCN and SCN. Three independent experiments were conducted to generate the data.

2.4.3 Immunochemistry

These experiments were performed to assess the morphological integrity of spinal cord neuron processes in the presence of glutamate, SCN, or OCN. Upon glutamate (30 µM), SCN (10 mM), or OCN (10 mM) application for 20 minutes, neuronal cultures were washed twice with the culture medium and left to recover for 24 hours before observing the tubulin network observed. Three independent experiments were conducted to generate the data. Neuronal cultures were fixed in 2% paraformaldehyde for 5 minutes, washed in PBS, and incubated in PBS-NH4Cl (100 mM) for 40 min, then permeabilized for 30 min in a blocking solution (0.25% fish gelatin with 0.2%Triton X-100 in PBS), and then for 24 h at 4°C with mouse IgG2a Anti-Beta-tubulin III Antibody (Clone TUJ1; 1:1000; ref 801201 BioLegend). The secondary antibody was diluted at 1/1000 in the blocking solution for 2h: Goat anti-mouse IgG2a Alexa 488 (1/1000; Invitrogen) + Hoechst for labeling nucleus (1/1000; ref H 33258, Sigma). Immunostaining was observed using a 63x oil-immersion objective with a numerical aperture of 1.32. Serial optical sections were obtained with a Z-step of 0.5 to 0.55 µm. Images (1024 x 1024; 12-bit color scale) were stored using Leica software LAS-AF and analyzed using ImageJ 1.5 software.

2.5. Statistical analyses

Data are presented as mean and standard error of the mean (SEM). Data were assessed for normality with the Shapiro-Wilk test. Accordingly, statistical comparisons for normally distributed data were performed using the Student's t-test (between two groups) or one-way ANOVA (for comparison between three groups or more). In the latter case, Holm-Sidak's test was used to correct for multiple comparisons. Corresponding non-parametric tests were applied in case data were not normally distributed (*i.e.*, the Mann-Whitney test, multi-comparison Kruskal-Wallis test, and Dunn's post hoc

comparison and correction for multiple comparisons). All statistical tests were two-tailed and performed at a 5% significance level. The half maximal inhibitory concentrations (IC50) of OCN and SCN in various conditions (cell lines and incubation time) were estimated by nonlinear regression using the logistic dose-response curve (or "[Inhibitor] vs. response" equation), with a 95% confidence interval (CI). The IC50 data are presented as IC50 in mM (lower - upper 95% CI limits). All statistical analyses were performed by GraphPad Prism Software version 7 for Windows (GraphPad Software, San Diego, California, United States) and R software version 4.3.2 for Windows (R Core Team, Vienna, Austria).

3. Results

3.1. Cell viability and underlying mechanisms

3.1.1. OCN, but not SCN, reduced Neuro-2A and SH-SY5Y cell viability

After 24-hour incubation, OCN significantly reduced cell viability in Neuro-2A cell lines at 0.3 mM and even more importantly at higher concentrations. Furthermore, a drastic and significant additional drop was observed between 24 and 72 hours at OCN concentrations ≥ 3 mM. Accordingly, OCN IC50 values also dropped with time (especially between 24 and 48 hours of incubation) (Figure 1a).

The SH-SY5Y cell viability significantly dropped after 24 hours only under OCN treatment at concentration ≥ 3 mM ($p < 0.001$ under 3, 10, and 30 mM OCN incubation, respectively) , and even further with longer incubation time (after 48 hours), with decreasing OCN IC50 values (Figure 1b).

In contrast, SCN-induced no reduction in Neuro-2A cell viability regardless of the concentration or the incubation time duration. Extremely high SCN concentrations (30 mM) were necessary to observe a modest cell death only after 48-hour incubation ($p = 0.664$). Strikingly, Neuro-2A cell viability even increased under SCN treatment at concentrations between 1 mM ($p = 0.022$) and 10 mM ($p = 0.001$) (Figure 2a). Reduction in SH-SY5Y cell viability was observed only at 30 mM SCN incubation for 24 hours ($p < 0.001$) and 48 hours ($p < 0.001$) (Figure 2b).

3.1.2. A glucose- and SAA-rich culture medium reduces OCN toxicity

After a 24-hour incubation in DMEM, OCN induced Neuro-2A cell viability drop (48.7 (6.6) %) only at high concentration (10 mM) in contrast with untreated cells ($p < 0.001$) and more prominently than in SH-SY5Y cells (15.1 (4.2) %; $p < 0.001$). When incubated in MEM, Neuro-2A and SH-SY5Y cells resisted worse to OCN toxicity at the same concentrations than in DMEM (Figure 3a). Accordingly,

the OCN IC50 values were higher in DMEM than in MEM for both Neuro-2A and SH-SY5Y cell lines (Figure 3b). Thus, DMEM significantly reduced the OCN toxicity in both Neuro-2A and SH-SY5Y cells.

3.1.3. OCN activates caspase-3, caspase-8, and caspase-9 in Neuro-2A cells

A 24-hour exposure of Neuro-2A cells to OCN 0.3 mM increased the caspase-3 activity (compared to controls) by 3.2 (0.4) folds ($p < 0.001$) (Figure 4a). Similarly, caspase-8 activity was increased by 2.5 (0.3) folds ($p < 0.001$) (Figure 4b), while caspase-9 activity increased by 2.1 (0.3) folds ($p = 0.004$) (Figure 4c). In summary, Neuro-2A cells susceptibility to OCN neuronal toxicity involved at least the three tested caspases.

3.1.4. OCN induces oxidative stress in Neuro-2A cells

The Neuro-2A cells submitted to OCN treatment yielded significantly higher levels of ROS as compared to untreated cells: 9.0 (0.8)-folds higher under OCN 0.3 mM ($p < 0.001$) (Figure 5). Thus, oxidative stress likely mediates at least partially the toxic effect of OCN in Neuro-2A cells.

3.2. Electrophysiological characterization of OCN and SCN excitotoxicity in spinal cord neurons

3.2.1. OCN, more than SCN, induces glutamatergic-like inward currents

Application of 30 µM Glu induced an inward current of much higher amplitude than under 10 mM SCN exposure (no effect) and 10 mM OCN (Figure 6a and 6b). While only four experiments (4/17) with SCN could evoke a current, three of them were observed with a delay that exceeded the duration of application. Therefore, SCN was considered to be poorly active in neuronal cultures through the glutamatergic pathway. On the other hand, OCN-induced currents increased in a dose-dependent manner from no current at 1 mM to -1074 (124) pA at 10 mM.

3.2.2. OCN-induced currents are mediated by ionotropic glutamate receptors

Because SCN evoked no significant response in cultured neurons (see above), we did not further test it in subsequent electrophysiological experiments. The simultaneous application of 20 µM CNQX and 100 µM d-AP5 significantly reduced glutamate-induced currents ($p = 0.001$) in a reversible way (Figure 7a). Application of both blockers almost completely abolished the OCN-induced response ($p < 0.001$) in a similarly reversible way (Figure 7b). The complete current block in the presence of

these ionotropic glutamate receptor antagonists highly suggested that OCN-induced currents were exclusively mediated by AMPA and NMDA receptors.

3.2.3 OCN may promote the release of glutamate at synapses

Glu and OCN may regulate the release of neurotransmitter from synaptic terminals. We thus analyzed the neuronal response to Glu and OCN in the presence of 1 µM TTX (which abolishes spontaneous excitatory synaptic transmission (Wakita et al., 2015)). Application of 30 µM Glu was still able to generate a current under TTX exposure ($p < 0.001$), whereas the OCN-induced currents were completely blocked ($p < 0.001$) (Figure 7). These results suggest that the OCN-induced response interfered with glutamatergic synaptic neurotransmission.

3.2.4. Short-term effects of Glu, SCN and OCN on neurites.

We finally evaluated Glu-induced neurotoxicity (i.e. excitotoxicity) on the neuronal integrity at short-term, in comparison with SCN and OCN toxicity. Upon Glu (30 µM) application, neurites were fragmented, whereas no apparent effect was observed with SCN (10 mM) and OCN (10 mM) (Figure 9). These results suggest that SCN and OCN were unable to elicit acute neurotoxic effects as fast as Glu.

4. Discussion

This study aimed at evaluating the neurotoxicological effects and mechanisms of SCN and OCN, two cyanide metabolites supposedly neurotoxic, hence being candidates as molecular actors in the occurrence of konzo, a spastic paraparesis of sudden onset consecutive to a selective upper motor neuron disease (Spencer, 1999). For this purpose, we performed experiments evaluating neuronal cell viability (including in a glucose- and SAA-rich environment) in two neuronal cell lines and investigated, among potential mechanisms, the activation of 3 caspases (caspase-3, caspase-8, and caspase-9), and oxidative stress. Additionally, electrophysiological measurements of glutamatergic currents were done in primary cultured spinal cord embryonic neurons to explore excitotoxicity, targeting specific glutamatergic ionotropic receptors and assessing the disruption of synaptic transmission.

The most important finding of our study is that OCN is more neurotoxic than SCN (which required concentrations well above physiologically encountered magnitudes and ingestion of extremely high amounts of improper cassava products, to induce cell death). Accordingly, OCN induced

glutamatergic like depolarizing currents, while SCN inconstantly induced extremely lower currents of similar patterns. Thus, OCN appears to be a more plausible neurotoxic compound than SCN, albeit probably less potent than glutamate (owing to lower elicited currents).

As a physiological metabolite of cyanide, OCN exists only in traces in the serum, which questions its effective neurotoxic role in vivo. However, OCN serum increase was documented upon cyanide exposure in experimental animals, especially when the latter were submitted to SAA-free diet (Tor-Agbidye et al., 1999). Thus, considering SAA deficit in konzo, the potential pathogenic role of OCN can be appropriately discussed. In addition, neuronal cell death induced by OCN could be rescued by a glucose- and SAA-enriched environment, which recalls the deleterious role played by SAA deprivation in konzo (Cliff et al., 1985) and potential protection by a balanced nutritional state (Baguma et al., 2021a). Moreover, OCN toxicity over neurons increased with time at unchanged concentration. This time-dependent enhancement of OCN effect is compatible with the long-lasting cassava-derived cyanide exposure undergone by affected population before appearance konzo outbreaks (Baguma et al., 2021a). OCN-induced cell death was also dose-dependent, featuring another pattern of konzo studies (showing increased cyanide exposure during konzo outbreaks) (Cliff et al., 1985).

From our data, OCN induced glutamatergic-like currents, which suggests excitotoxic mechanisms, in case these currents are involved in OCN-induced neurotoxicity. In this regard, both AMPA and NMDA receptors seemed to be involved. The complete inhibition of OCN-induced currents by blockers of these two receptors suggests that metabotropic receptors are not implicated in OCN-mediated excitotoxicity, unless their activation requires much higher OCN concentrations than those applied in our experiments. In contrast, glutamate effect persisted beyond AMPA and NMDA blockade. Complete inhibition of OCN currents by TTX application suggests that OCN does most probably not have a direct effect on glutamate receptors, rather it interferes with glutamatergic synaptic neurotransmission.

The presynaptic glutamate release may lead to an accumulation of glutamate in the synaptic cleft, resulting in excessive activation of post-synaptic glutamate receptors, which, in turn, may induce neuronal damage via excitotoxicity (Van Damme et al., 2005b; Wang and Qin, 2010). Excitotoxicity has been associated with other motor neuron diseases such as amyotrophic lateral sclerosis (Van Damme et al., 2005b, 2005a) and neurolathyrism (Heath and Shaw, 2002; M. Van Moorhem et al., 2011). The latter, caused by the consumption of a grass pea (Lathyris sativum), shares the same clinical picture of a sudden-onset spastic paraparesis with konzo (Ludolph and Spencer, 1996). Beta-

N-Oxalylamino-L-alanine (L-BOAA), a stereospecific agonist of AMPA receptors contained in the grass pea, was identified as the causal agent of neurolathyrism (Ngudi et al., 2012; Spencer and Palmer, 2012). Through experimental studies in rodents, L-BOAA was shown to induce upper motor neuronal death through excitotoxic AMPA receptor activation (M. Van Moorhem et al., 2011). This is believed to be the mechanism leading to spastic paraparesis in neurolathyrism (Ross et al., 1989; Spencer, 1999; M. Van Moorhem et al., 2011; Marijke Van Moorhem et al., 2011). By analogy, we can postulate that OCN activation of AMPA receptors participates to OCN-induced neuronal death. Moreover, the OCN-induced excitotoxic effect would extend to NMDA receptors, given their activation upon OCN application.

We observed that OCN increases ROS levels, showing involvement of oxidative stress pathways. Previous studies have documented prooxidant effects of OCN (Hu et al., 2019; Sokołowska et al., 2013). For instance, experimental rats displayed a significant increase of free radicals in the brain cortex, hippocampus, and striatum, 2.5 hours after intraperitoneal administration of OCN (Sokołowska et al., 2013), in support to our findings. OCN-induced oxidative cell damage may result either in necrosis or apoptosis (Kannan and Jain, 2000). The activation of Caspase-3, 8, and 9 observed in response to OCN application supports apoptosis as a possible mechanism of OCN-induced neurotoxicity. Caspases involved in apoptotic processes are classified in two sub-groups: apoptosis initiating caspases (2, 8, 9, and 10) and effector caspases (3, 6, and 7) (Shi, 2002). Once activated by diverse proapoptotic inducers (e.g. intracellular calcium accumulation, prooxidant agents, ionizing radiations, ischemia, drugs, organic solvents and metabolites, etc. (Kannan and Jain, 2000)), initiator caspases are responsible for the cleavage and activation of downstream effector caspases. The latter, in turn, are responsible for DNA fragmentation and downstream cascades, which are hallmarks of apoptosis (Fulda and Debatin, 2006; Srinivasula et al., 1998).

Our data show the implication of apoptosis related to 3 caspases (3, 8, and 9). Initiator caspases are mainly activated either by an extrinsic pathway (involving cell surface receptors, which initiate Caspase-8 activation), or by an intrinsic pathway (involving outer mitochondrial membrane permeabilization and apoptogenic proteins release responsible for Caspase-9 activation) (Fulda and Debatin, 2006). Thus, our results are compatible with OCN-induced simultaneous activation of Caspase-8 and Caspase-9, in addition to the Caspase-3-mediated effector pathway. In addition, OCN could indirectly activate the caspase pathway via ROS production.

Overall, OCN-induced neurotoxicity seems to be mediated by several concomitant mechanisms. To the best of our knowledge, this is the first study experimentally documenting the toxic effect of OCN

directly on neuronal cells. Beyond the three investigated pathways, OCN neurotoxicity could also be mediated by post-transcriptional protein modifications through the carbamylation process (Badar et al., 2019). Indeed, there are some indications from proteomic experiments, that OCN could be neurotoxic through carbamylation of albumin and other spinal cord proteins, especially in a SAA-deprived environment (Kassa et al., 2011; Kimani et al., 2013). Previous f (Oluwole and Oludiran, 2013), even if no causal relationship has been established between konzo and elevated SCN, as both konzo patients and healthy subjects living in konzo-affected areas usually have similarly high SCN levels (Baguma et al., 2021b; Banea et al., 1997; Tylleskar et al., 1992). SCN is deemed to be much less toxic than cyanide (Pimenta et al., 2010; Schulz, 1984) and than OCN (as observed in our data), but it is possible that SCN neurotoxicity only appears in case of excessive accumulation, with confusion, hyperreflexia, and convulsions (Hollenberg, 2007) witnessing central nervous system dysfunction (Smith, 1973). Thus, as found in our study, only very high SCN concentrations are susceptible to be toxic to neuronal cells. Of notice, the SCN concentration used in these experiments is more than 100-fold higher than the normal plasmatic levels (68.8 - 344 µmol/L (Charlier et al., 2000)) and up to 30-fold higher than the concentrations usually found in populations exposed to cassava-derived cyanide poisoning (below 1 mmol/l), in contrast with OCN-induced toxicity, which appears to be engaged at lower concentrations (0.3 mM). In support to our assumption, and based on the equation by Cardoso et al. (2004), one should daily ingest 20 to 60 kg of poorly processed cassava flour containing 10 to 30 mg of cyanide per kg (Baguma et al., 2022) in order to reach a 10 mM SCN concentration in vivo (which was not enough to reduce neuronal viability in our experimental settings), but which is well above the micromolar range of SCN concentrations in biological fluids in healthy individuals (Chandler and Day, 2015), but also in konzo-diseased patients (Banea et al., 1992; Kambale et al., 2017). Knowing, in addition, that the usual amount of cassava consumed daily remains < 1 kg (Adebayo, 2023; Burns et al., 2010; De Moura et al., 2015), it realistically appears impossible either to reach such SCN levels under physiological conditions or to consume the amount of improper cassava needed for SCN to become neurotoxic. .

This highly questions SCN-induced neurotoxicity, particular through excitotoxicity, unless much lower concentrations are sufficient to induce neuronal death in vivo. So far, we have no indication for such a scenario. Against this assumption, several studies have previously reported protective effects of SCN against oxidative damages and positive effects on host defenses against infections (Chandler et al., 2013), on the inflammatory status in mouse models of cardiovascular diseases (Morgan et al., 2015; Zietzer et al., 2019), as well as a protective effects against the deleterious effects of myocadiac infarct in rats (Hall et al., 2021) and even in humans (Nedoboy et al., 2014).

Accordingly, Neuro-2A cells treated under SCN 1 mM to 10 mM had even a higher viability rate compared to untreated cells, to our surprise.

Alternatively, it is also possible that combined toxicity of SCN and OCN causes konzo, as suggested in one old study (Smith, 1973), and by similar postulated glutamatergic mechanisms from our results (despite minor SCN-elicited currents). In this study, both SCN and OCN induced depolarizing currents in primary rat spinal cord neurons through activation of glutamate receptors. Smith R. had already hypothesized that OCN and SCN could act on the same receptors or sites in the central nervous system as their toxicity shares a similar clinical picture. So far, we have no indication of such a scenario.

Overall, the toxic effect of OCN appears multiple and intricated, plausibly resulting in upper motor neuronal damage and subsequent death, and ultimately causing konzo. This innovative conceptual hypothesis about konzo etiology deserves attention for future studies. Namely, whether OCN action is direct or indirect, results from other intermediated compounds, and how these mechanisms are triggered, timely engaged, and associated, remain open questions to be elucidated.

5. Conclusion

Our results suggest OCN as a neurotoxic agent, while SCN toxicity could be questioned at such high concentrations. Furthermore, the gradual–OCN toxicity and its enhancement in a SAA-poor environment are compatible with konzo, especially knowing that OCN synthesis results from cyanide metabolization only in SAA-deprived conditions and that konzo has exclusively been reported in nutritionally compromised subjects. OCN interferes with several pathways, including excitotoxicity, oxidative stress, and apoptosis, which suggests that several mechanisms are involved in OCN-induced neurotoxicity. The results obtained in this study opens new perspectives for a better understanding of the pathogenesis of konzo and other related motoneuron diseases.

Acknowledgement

This work was funded by the VLIR-UOS Team Project 2017 (Ref. No. CD2017TEA439A104), by the Board of Directors of UHasselt (Special Research Fund, BOF 2017 - Bilateral Scientific Cooperation. Ref: BOF17BL19), and by the Doctoral Schools of UHasselt (Doctoral Schools Mobility grant - call 1 2020). We thank Susanne Bolte, Jean-François Gilles, and France Lam for assistance with confocal imaging (IBPS imaging facility).

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Figures

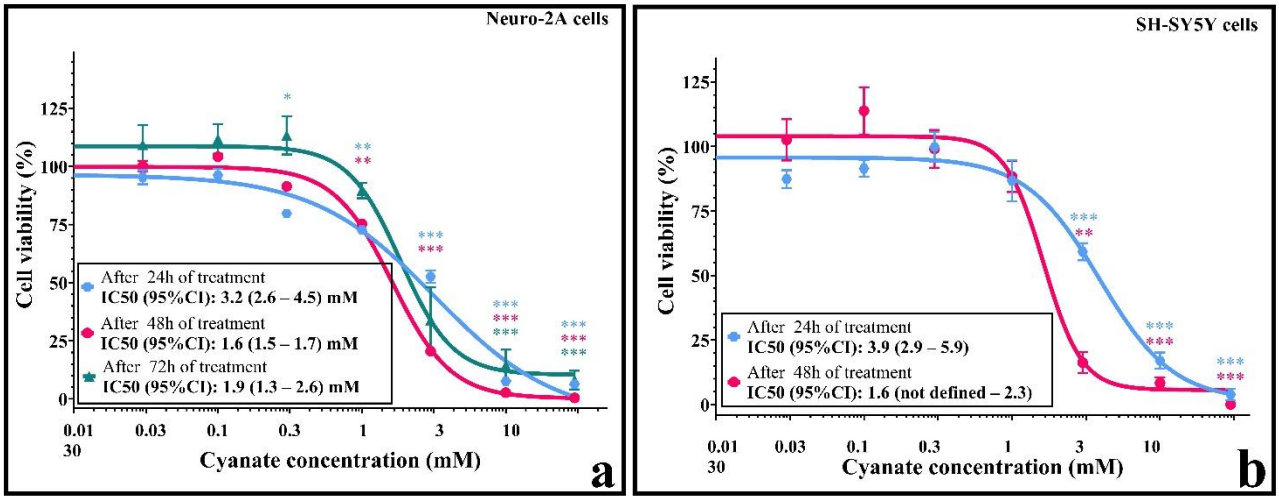

Figure 1. Neuro-2A and SH-SY5Y cell viability under OCN treatment in MEM medium. Cell viability was measured after 24 hours (blue), 48 hours (red) and 72 hours (green) of treatment with increasing OCN concentrations in MEM culture medium. Data were obtained from three independent experiments and are plotted as mean and standard error of the mean (SEM). **(a) Neuro-2A cells** (sample size (n) = 350 quadruplicates). When cells were incubated with OCN for 24 hours at 0.3 mM, their viability dropped at 80.2 (2.1) %. It dropped even more at 10 mM and 30 mM (7.9 (0.5) %) and 6.8 (0.4) % respectively). Moreover, an additional reduction was observed with longer incubation time at OCN concentrations ≥ 3 mM, with IC50 values (95% CI)) of 3.2 (2.6 - 4.5) mM, 1.6 (1.5 - 1.7) mM, and 1.9 (1.3 - 2.6) mM respectively after 24h, 48h, and 72h of treatment. **(b) SH-SY5Y cells** (n = 244 quadruplicates). The cell viability dropped respectively to 59.2 (3.4) %, 17.0 (3.2) %, and 3.8 (2.2) % under 3, 10, and 30 mM OCN exposure. It lowered even further with longer OCN incubation time: after 48 hours, it dropped to 16.2 (4.1) %, 8.3 (2.2), and 0.0 (0.0) % respectively under OCN 3, 10, and 30 mM. The IC50 values of OCN were 3.91 mM (95% CI: 2.89 - 5.985 mM) and 1.63 mM (95% CI: not defined - 2.326 mM) respectively after 24h and 48h of treatment. The Kruskal-Wallis test was applied, followed by the Dunn's Multiple Comparison post hoc test between treated and untreated cells of the same incubation time. * denotes significant difference with $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$. IC50 (95% CI): the half maximal inhibitory concentrations with the corresponding 95% confidence interval.

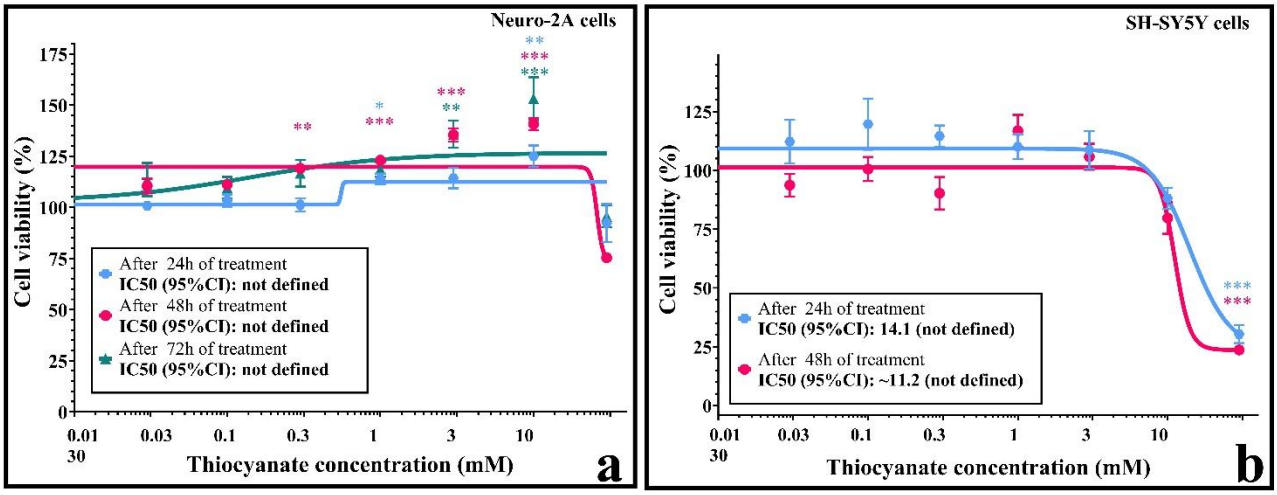

Figure 2. Neuro-2A and SH-SY5Y cell viability under SCN treatment in MEM medium. Cell viability was measured after 24 hours (blue), 48 hours (red) and 72 hours (green) of treatment with increasing SCN concentrations in MEM culture medium. Data from three independent experiments are plotted as mean and standard error of the mean (SEM). **(a) Neuro-2A cells** (n = 331 quadruplicates). No reduction was observed in cell viability, except at 30 Mm and only after 48h incubation (cell viability = 75.4 (2.0) %, p = 0.664). At the contrary, there was an increase in cell viability under SCN treatment at concentrations between 1 mM and 10 mM. **(b) SH-SY5Y cells** (n = 256 quadruplicates). A small cell death was observed only at 30 mM OCN incubation (cell viability = 30.4 (3.8) % after 24 hours and 23.6 (2.1 % after 48 hours, $p < 0.001$), with IC50 respective values of 14.1 mM (95% CI not defined) and approximately 11.2 mM (95% CI not defined). Statistical tests were performed using the Kruskal-Wallis test followed by Dunn's Multiple Comparison post hoc test. * denotes a significant difference with $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$ between treated and untreated cells of the same incubation time. IC50 (95% CI): the half maximal inhibitory concentrations with the corresponding 95% confidence interval.

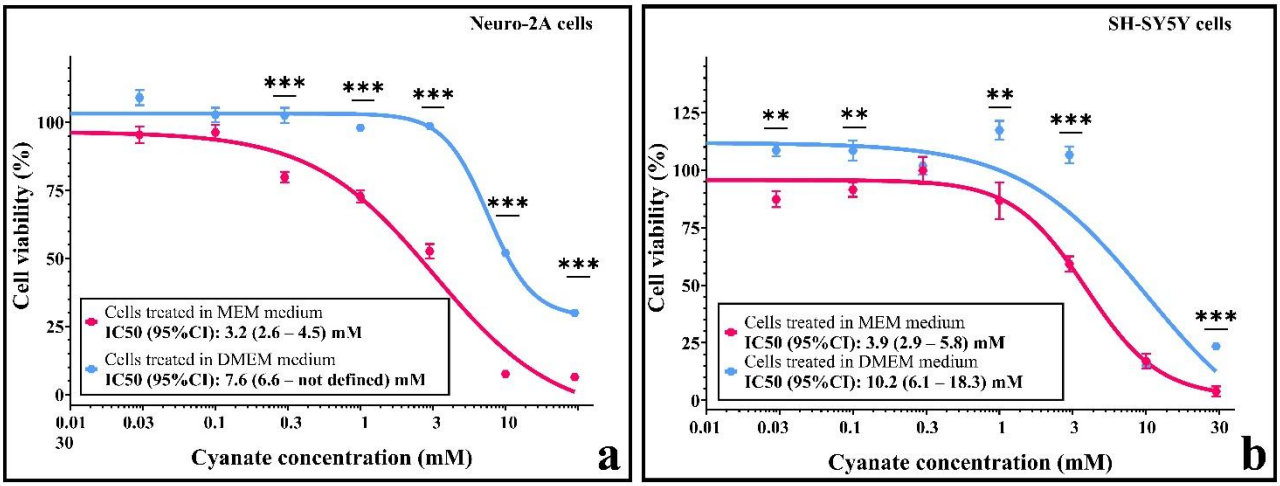

Figure 3. OCN toxicity in Neuro-2A and SH-SY5Y cells cultured in MEM versus in DMEM. Data are plotted as mean and SEM. **(a) Neuro 2A cells** (n = 220 quadruplicates; 80 for DMEM and 140 for MEM experiments). In DMEM medium (blue), cell viability dropped with increasing OCN concentrations (respectively 98.7 (1.5) %, 51.9 (1.5) %, and 30.0 (1.2) % at 3 mM, 10 mM, and 30 mM). Cell viability was worse in MEM medium (red): 79.8 (2.0) %, 72.7 (2.2) %, 52.6 (2.6) %, 7.6 (0.5) %, and 6.4 (0.6) % at 0.3 mM, 1 mM, 3 mM, 10 mM, and 30 mM, respectively). Accordingly, the IC50 was lower in MEM than in DMEM. **(b) SH-SY5Y cells** (n = 187 quadruplets; 64 for DMEM and 123 for MEM experiments). Cell viability in DMEM dropped only at OCN concentrations ≥ 10 mM (respectively 15.1 (1.5) % and 23.4 (1.7) % at 10 mM and 30 mM). The drop was again more pronounced in MEM medium (respectively 86.7 (8.0) %, 59.2 (3.4) %, 17.0 (3.2) %, and 3.8 (2.2) % at 1 mM, 3 mM, 10 mM, and 30 mM) and the IC50 lower. Comparisons between cell viability in DMEM versus MEM medium at the same OCN concentration were done using the Mann-Whitney U test. ** denotes a significant difference with $p < 0.01$ and *** for $p < 0.001$. IC50 (95% CI): the half maximal inhibitory concentrations with the corresponding 95% confidence interval.

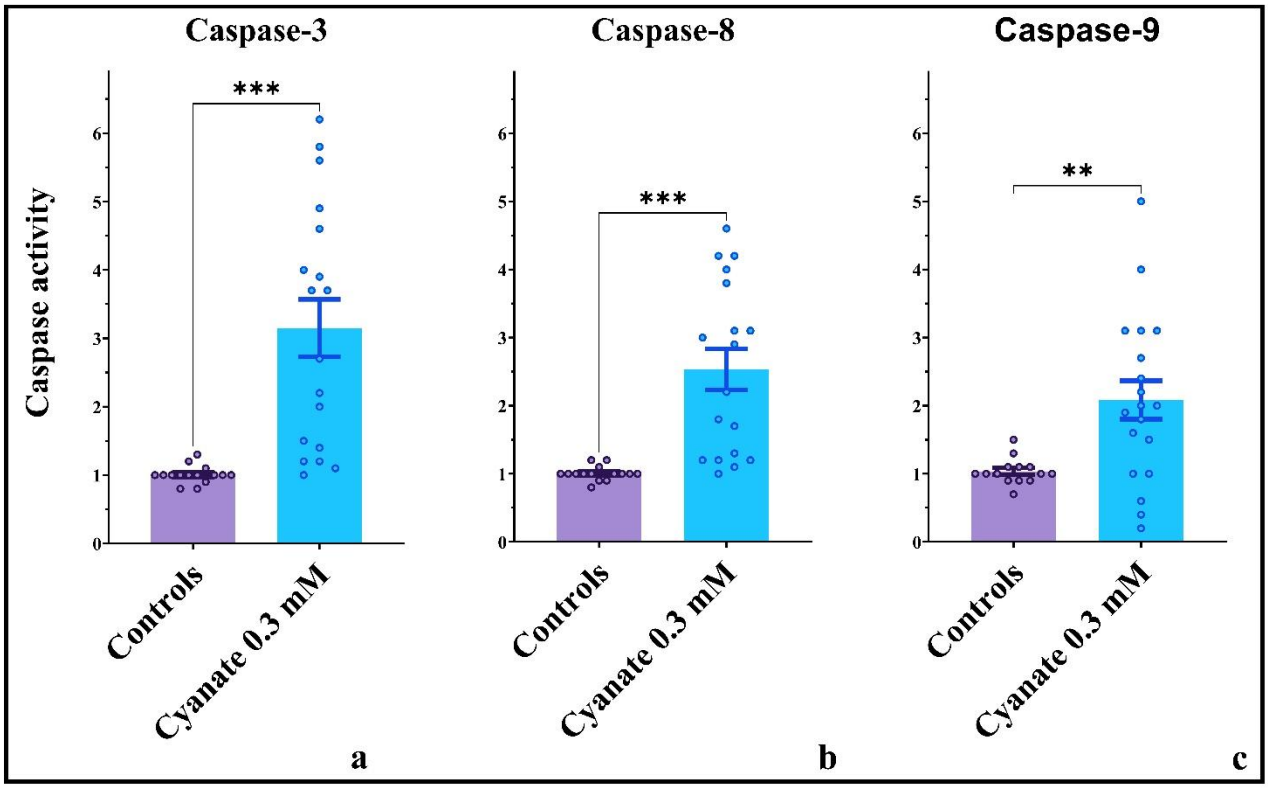

Figure 4. Effects of OCN treatment on caspase activities in Neuro-2a cells. Caspase-3 (a), **caspase-8 (b),** and **caspase-9 (c)** activity after 24 hours of OCN treatment was normalized to the values of controls (untreated cells). For each caspase, n = 15 in control conditions (purple) and n =18 for OCN 0.3 mM exposure (blue). Data are plotted as mean and SEM (three independent experiments) and analyzed using the Mann-Whitney test for Caspase-3 and Caspase-8, and the unpaired t-test for Caspase-9. ** denotes significant difference with $p < 0.01$ and *** denotes $p < 0.001$, compared to untreated cells.

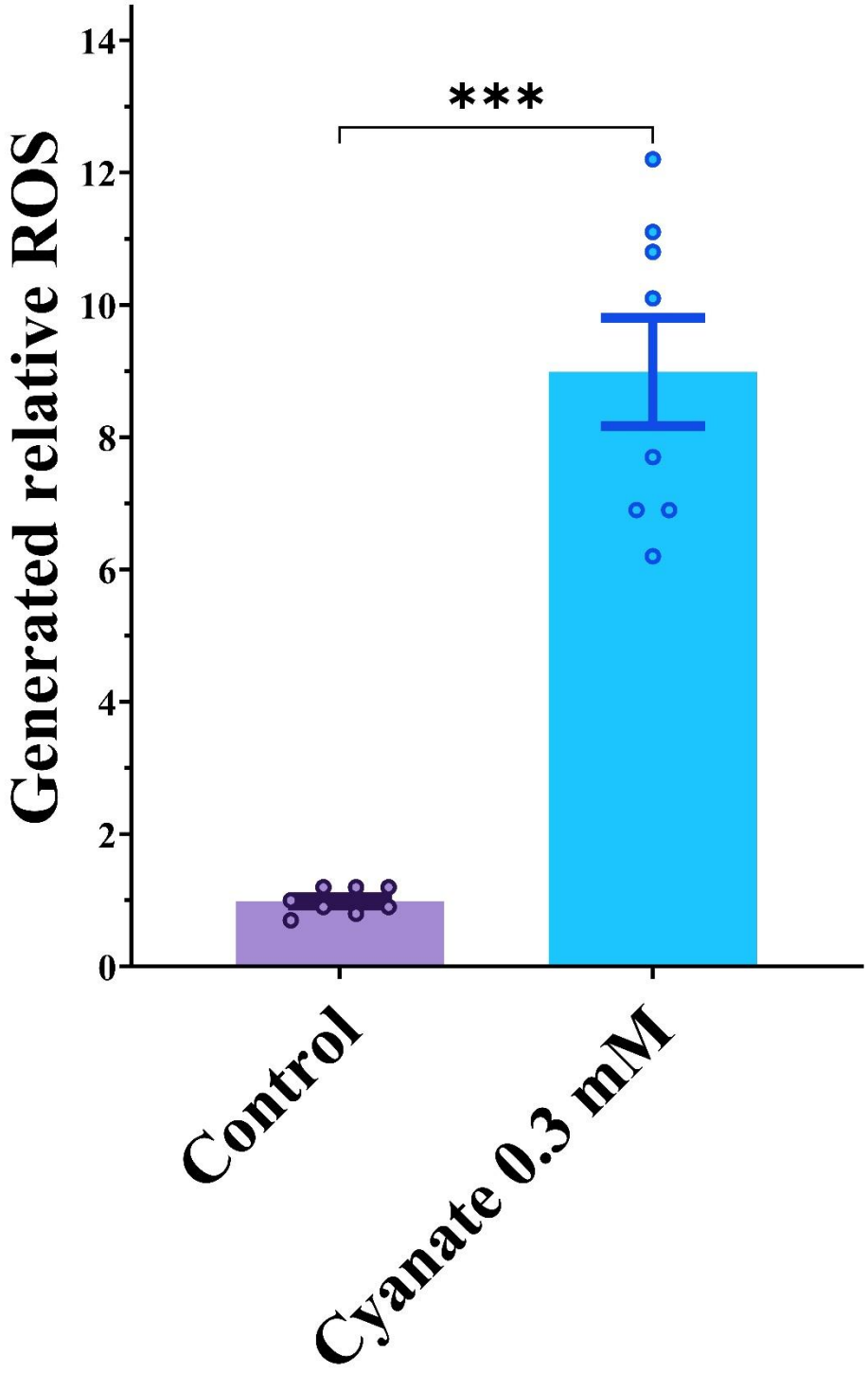

Figure 5. Effects of OCN treatment on mitochondrial reactive oxygen species (ROS) generation in Neuro-2A cells. n = 8 for control conditions (purple) and n = 8 for OCN 0.3 mM treatment (blue). Data from two independent experiments are plotted as mean and SEM and analyzed with an unpaired t-test. *** denotes a significant difference at $p < 0.001$ when comparing treated to untreated cells.

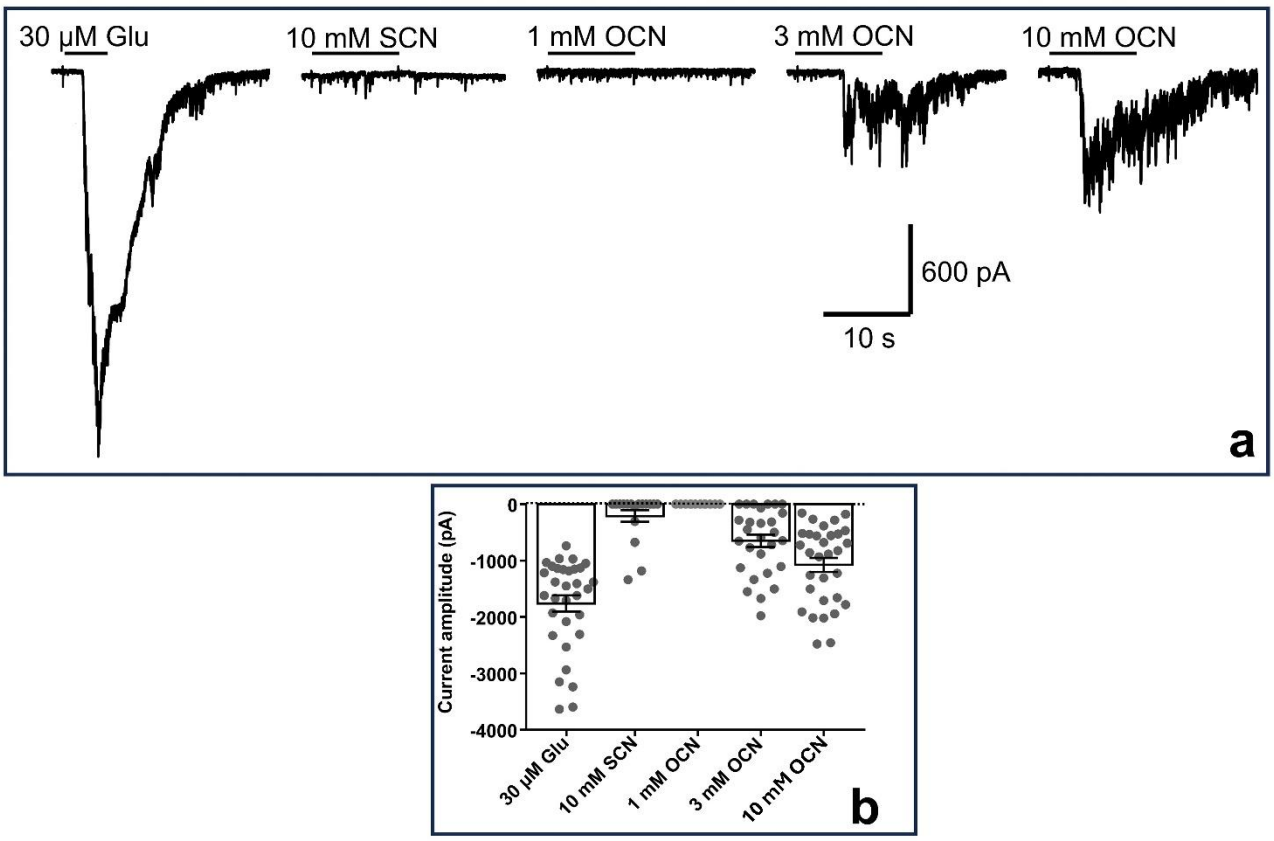

Figure 6. Electrophysiological responses induced by the application of Glu, SCN and OCN in primary spinal cord neurons. Data are plotted as means and SEM **(a)** Currents induced by Glu (30 µM) were of much higher amplitude than OCN-induced current at 10 mM. Further, from no current at 1 mM, currents elicited by OCN increased with OCN concentrations through 3 mM up to 10 mM. **(b)** The amplitude of currents induced by 30 µM Glu (n=32) equaled -1758 (143) pA, much higher than the SCN-induced current at 10 mM (-208 (105) pA, n = 17), and than 10 mM OCN (n=31; -1074 (124) pA). While 1 mM OCN (n = 12) had no effect, 3 mM (n = 28) and 10 mM OCN (n=31) evoked -649 ± 110 pA (n = 28) and -1074 ± 124 pA respectively.

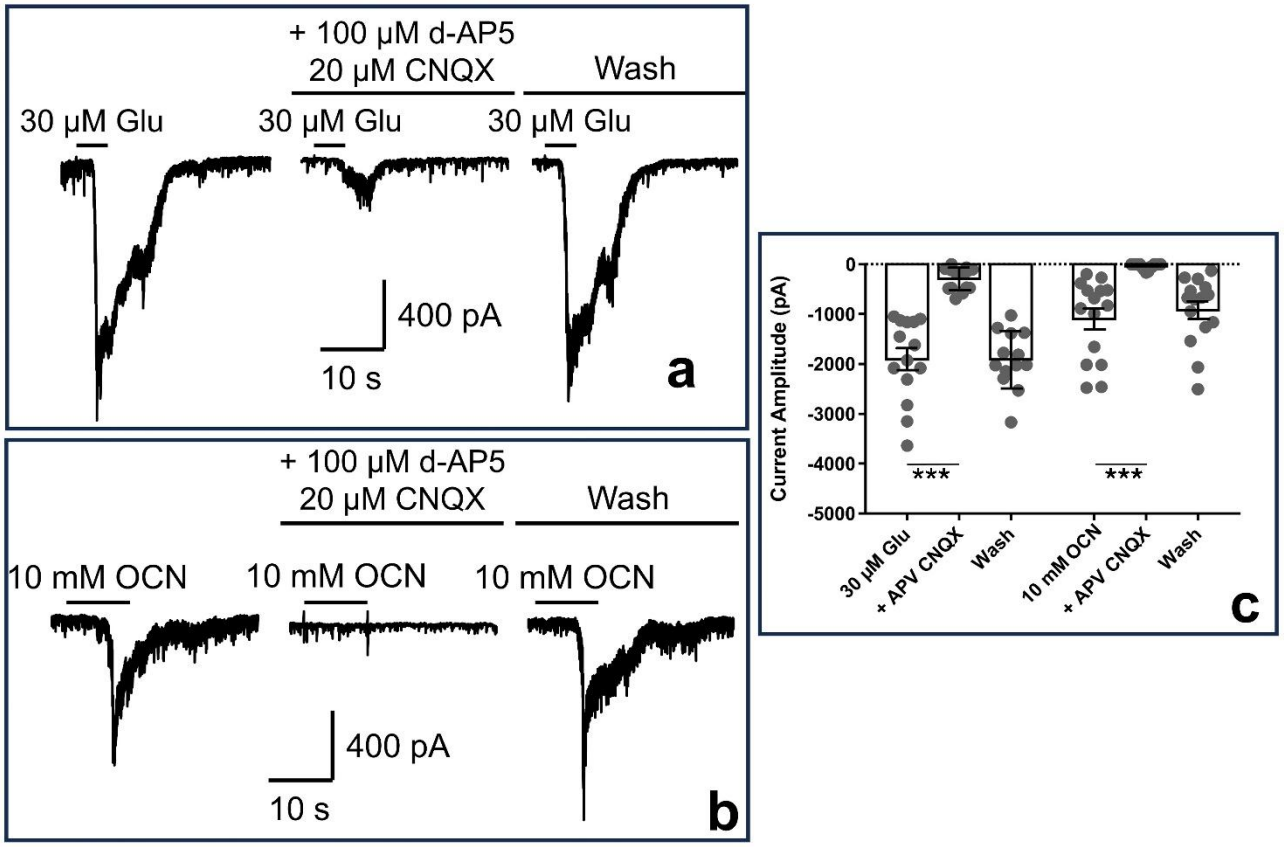

Figure 7. Effects of d-AP5 and CNQX application on the glutamate- and OCN-induced electrophysiological responses in primary spinal cord neurons. Data are plotted as means and SEM**. (a)** The inward current induced by Glu 30 µM was completely blocked by the concomitant application of d-AP5 (100 µM) and CNQX (20 µM), but this inhibition was reversible upon washout. **(b)** the same experiment replicated with OCN showed complete abolition of OCN-induced inward current, which was still reversible after washing. **(c)** The amplitude of currents induced by 10 mM glutamate (n=14) equaled -1905 (223) pA, was reduced to -289 (60) pA upon d-AP5 and CNQX application and increased back to 1911 (160) pA after washout. The same observation was made after OCN (n=14) application: d-AP5 and CNQX reduced the inward current from -1096 (208) pA to -36 (14) pA and washing out restored it to 921 (175) pA. Data comparisons were performed using the paired t-test. *** denotes a significant difference with $p<0.001$ when comparing values before and after d-AP5 and CNQX application.

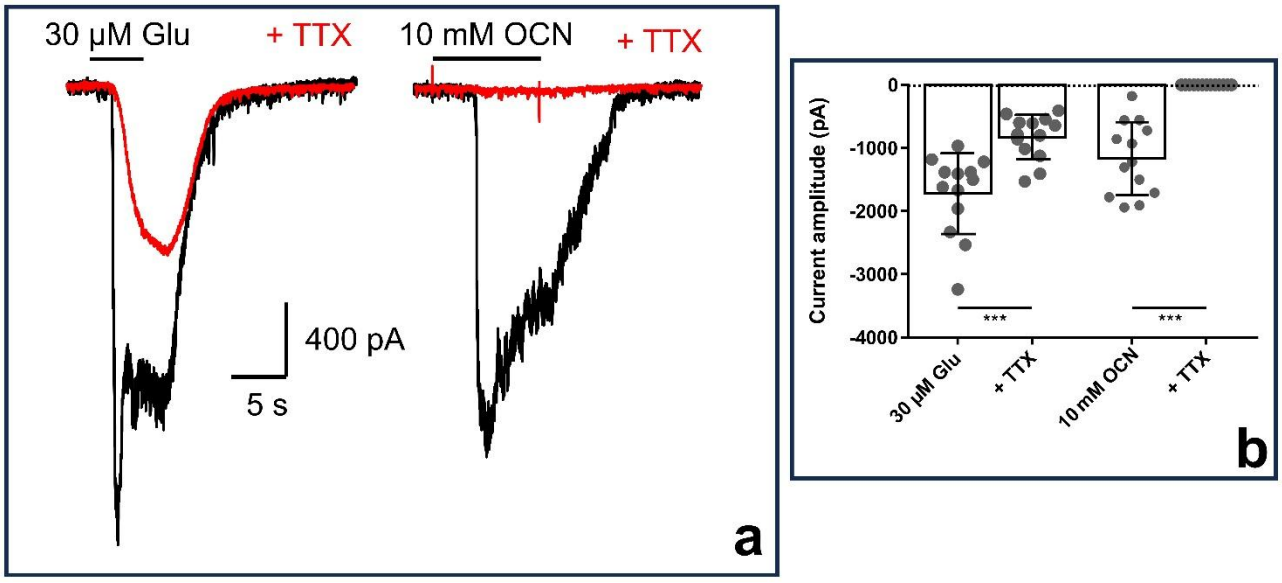

Figure 8. Effects of application of TTX on the glutamate and OCN-induced electrophysiological responses in primary spinal cord neurons. (a) Currents induced by Glu or OCN (black curves, left and right traces respectively) were partially (for Glu) or totally (OCN) abolished in the presence of TTX (red curves). **(b)** Quantitative data showed that Glu-induced currents were reduced by TTX application from (from -1722 (177) pA to -828 (97) pA, n=13, $p < 0.001$), while OCN currents were completely abolished (from -1168 (160) pA to 0.0 (0.0) pA, n = 13, $p < 0.001$). Data are plotted as means and SEM and compared (before and after TTX exposure) by the paired t-test. *** denotes a significant difference with $p<0.001$.

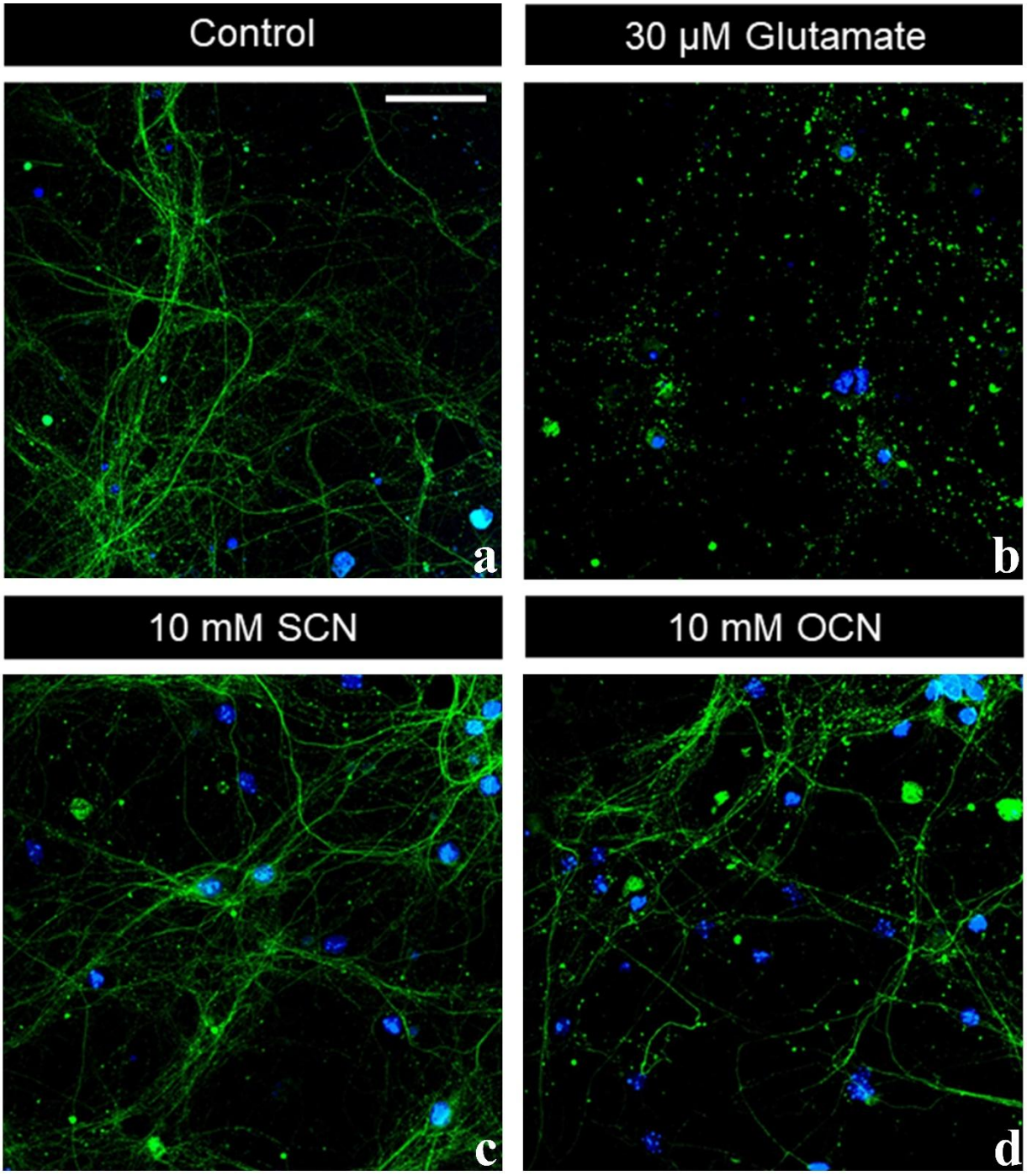

Figure 9. Glu but OCN and SCN induced neuronal damage. (a) In the absence of drugs (Control), neurites were easily observable using immunodetection of tubulin III. After 20 min of application, followed by a wash-out and 24 hours of recovery, Glu 30 µm induced visible neurite fragmentation **(b)**, contrary to 10 mM SCN that showed no visible effect **(c)**. 10 mM OCN showed a start of a fragmentation process **(d)**.