Neurotoxicity Study Design: The Essential Element of Sacrifice Times

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The Time Course of Neuronal Degeneration Should Guide the Timing of Assessment

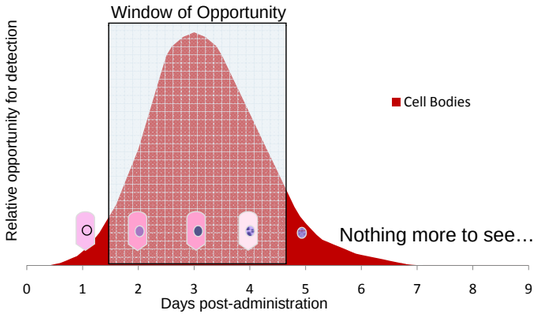
With the burgeoning number of neuroactive compounds being created, be they drugs or chemicals introduced into the environment, it is vital to public health that adequate means of assessing neurotoxicity are utilized. Fortunately, new technologies are not required, as there is a wealth of information and tools embedded in the history of neuroscience. **The fundamental objective measure of neurotoxicity is to measure cell death in the brain following administration of the potential toxin.** There are three elements critical for adequate detection of potential neurotoxicity: 1) schedule of sacrifice times following insult or exposure, 2) sampling intervals within the brain, and 3) means of detection. Of these, the first is typically not implemented effectively in toxicology studies and the second needs significant overhaul.

Although extensively documented in earlier literature, it is generally unappreciated that **the debris associated with neurodegeneration does not persist indefinitely and is cleared away from the nervous tissue**. The time course for the degeneration of neuronal components was worked out in the course of studies in the 1960s and 70s by researchers tracing axon pathways in the brain (utilizing deliberately-created lesions and varied time-points of sacrifice). From those studies we know there are **consistent windows of opportunity for witnessing the degeneration of the different neuronal components**. Evidence of degenerated synaptic terminals appear the earliest and disappear the quickest (all within a 2-3 day span); the debris of dendrites and cell bodies is cleared by 5 days post-insult; and axonal debris -persisting the longest- is gone by 7 days. For forms of insult that result in delayed neurotoxicity (e.g. certain organophosphates) the disintegration process is delayed but, once started, follows the same time course. If the test animal is sacrificed too soon or too late, the degenerative debris is not visible.

Neurotoxicity study designs have traditionally included dosing regimens of 14 or 28 days followed by sacrifice, however, this may not include adequate windows of opportunity for observing degeneration in any of the neuronal elements. Today, we can use information gleaned from historical studies and implement similar techniques to detect degeneration caused by neurotoxins. Here we present degeneration profiles for several known neurotoxins and show how essential sacrifice times are for accurate neurotoxicity testing.

Temporal Characteristics of Cell Death Following Acute Exposure

Cellular changes associated with cell death are visible in neurons ~2-5 days after exposure to an acute neurotoxin



Following acute neurotoxicity, a general screening staining method such as H&E (hematoxylin and eosin) detect pyknotic nuclear changes in neurons (condensation of chromatin in the nucleus of a dying cell is represented in this cartoon by dark blue-ish nucleus) and karyorrhexis (fragmentation of the nucleus is represented here by a blue-ish speckled nucleus). There is a limited window of opportunity in which to observe these changes.

Summary of Concepts Gathered from Past Research

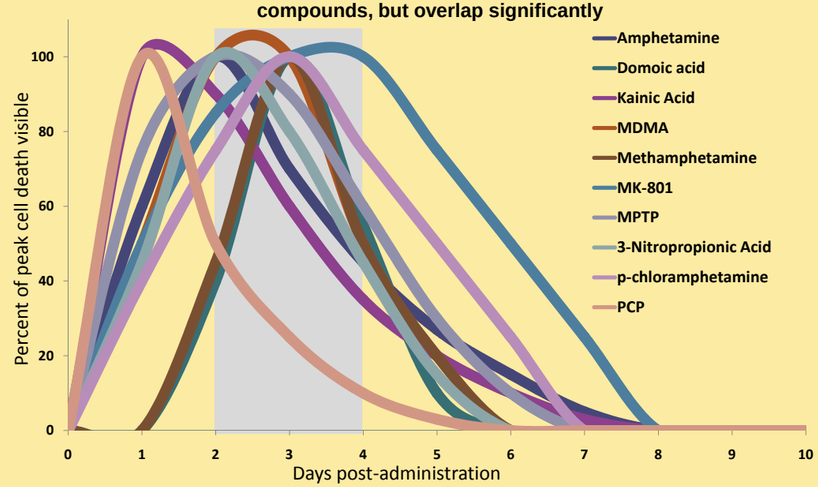
- All cells vulnerable to a compound tend to begin dying at the same time
- This cell death pattern begins within 1-5 days after acute administration
- The peak observation opportunity for cell death is 2-5 days after dosing
- By 5-10 days, no evidence exists that cell death occurred

The Window of Opportunity to Observe Peak Cell Death is Usually ~2-4 Days Post-administration

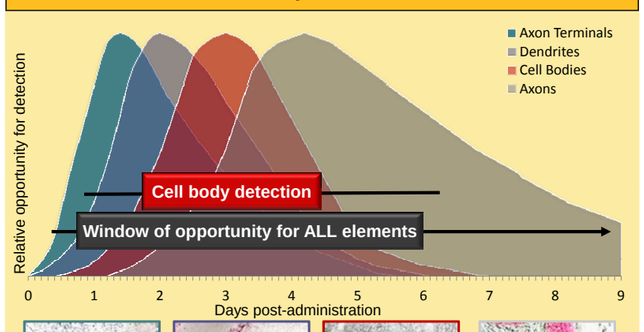
The evidence of cell death is a transient event in the brain. Unlike other organs, it is difficult to observe cell death after it has occurred because (1) debris from dead and dying cells is rapidly cleared from the brain by macrophagic microglia and (2) surviving neurons and glia can 'close ranks' leaving no 'empty spaces' (unless the lesion is huge, of course). ***While the observable evidence is transient, the effects of cell death are permanent.***

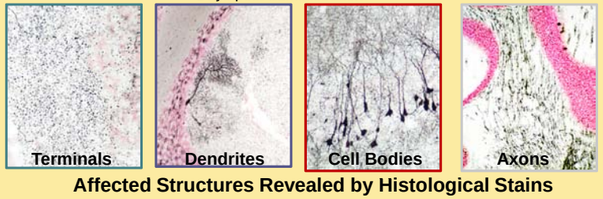
Figure legend: Timing profiles of neurodegeneration have been described by many researchers, however a comparative study has not previously been done to our knowledge. Here we overlay known timing profiles for 10 neurotoxins to illustrate that despite variations between compounds, acute neurotoxic effects can be observed in the same narrow window of time for all of them.

Cell death in known neurotoxins has been found to vary between compounds, but overlap significantly



Disintegration of each Neuronal Element Has Predictable Temporal Characteristics





Affected Structures Revealed by Histological Stains

STAIN	Neuronal Soma	Axons	Dendrites	Axon Terminals
CuAg Methods	X	X	X	X
FluoroJade	X	X	X	X
TUNEL	X			
Nissl	X			
H&E	X			

- Many histological staining methods indicate somatic damage.
- Neurodegeneration stains (*e.g.*,amino cupric silver or FluoroJade) can detect pathological changes involving all elements of the neuron.
- Neurodegeneration stains provide a **longer window of opportunity** to view death of the neuron.

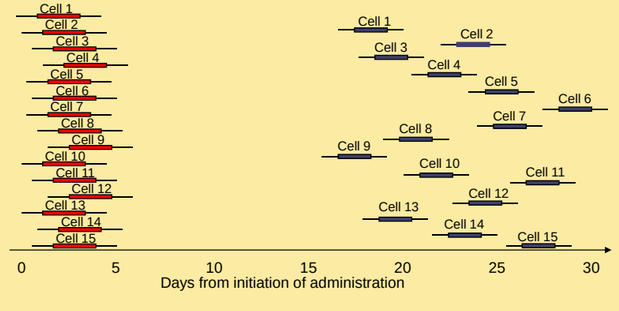
Sub-Chronic and Chronic Neurotoxicity Testing

- Subchronic and chronic dosing/exposures are relevant to potential new drugs and environmental agents.
- Neurotoxic effects after acute dosing are revealed at early time points.
- The potential neurotoxic affects following repeated sub-chronic and chronic dosing/exposure are likely to be broadly distributed over time and require a broader range of sacrifice times.

Fewer observables of cell death exist at any given time with sub-chronic and chronic exposures

Acute Neurotoxicity: all affected cells die within a short window of time.

Sub-chronic and Chronic Neurotoxicity: cell death is scattered across time.



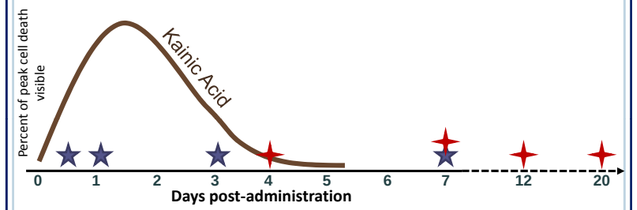
Decreased evidence of neurotoxicity (fewer observables) at any given time point under repeated dosing conditions requires a wider range of sacrifice times as well as a means of detection that captures all elements of the neuron (*e.g.*, amino cupric silver, FluoroJade).

Assessing Neurotoxicity at the Wrong Time Can Lead to False-Negative Results

Example

Study 1: C57BL/6 and BALB/c mice did NOT exhibit excitotoxic cell death in limbic structures at any of the time points examined (4, 7, 12, 20 days post-administration), leading to a conclusion that these animals were genetically resistant to kainate-induced neurotoxicity.
Schauwecker & Steward (1997). Proc. Natl. Acad. Sci. USA. Vol. 94 pp. 4103-4108.

Study 2: C57Bl/6J exhibited extensive cell death in limbic structures at 12 and 24 hrs following kainic acid administration, which was dramatically attenuated by 3 days post-administration. These mice are NOT resistant to kainate-induced neurotoxicity damage. Benkovic, O'Callaghan & Miller (2004) Brain Research. Vol. 1024. pp. 59-76.



The probability of observing damage induced by kainic acid (brown curve) peaks around 2 days post-administration and is negligible after 4 days. In the first study neurotoxicity was assessed at time points too late to witness the damage and the results were incorrectly interpreted.

Making assessments too early or too late can lead to incorrect answers. Until studies have been done to describe the full timing profile of a neurotoxin, a given result can only be valid for the time-point assessed.

Recommended Sacrifice Schedule

Days post-administration	Acute Neurotoxicity Assessment	Sub-Chronic Neurotoxicity Assessment	Chronic Neurotoxicity Assessment
2-3	X	X	X
5	X	X	X
9-10		X	X
16-20		X	X
25-30		X	X
60			X
90			X
180			X
270			X
360			X

- Select sacrifice times that fall within the time period during which disintegrative degeneration occurs (window of opportunity) as a function of acute or repeated dosing.
- Windows of opportunity are expanded by the use of a neurodegeneration stain that reveals all parts of the neurons.

For more information and complete references, please see:
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