

# Essential Elements and Considerations for Neurotoxicity Study Designs

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# Agenda

- ▶ Introduction
- ▶ Baseline and stepwise approach to testing
- ▶ Introduction of staining techniques
- ▶ Baseline evaluation principles:
  - ▶ Location/sampling
  - ▶ Timing/sacrifice dates
  - ▶ Stains
  - ▶ Study variables and modifiers
- ▶ Other types of assessments
- ▶ Protocol Summaries
- ▶ Questions/discussion

# Neurologic safety screens present a unique set of challenges

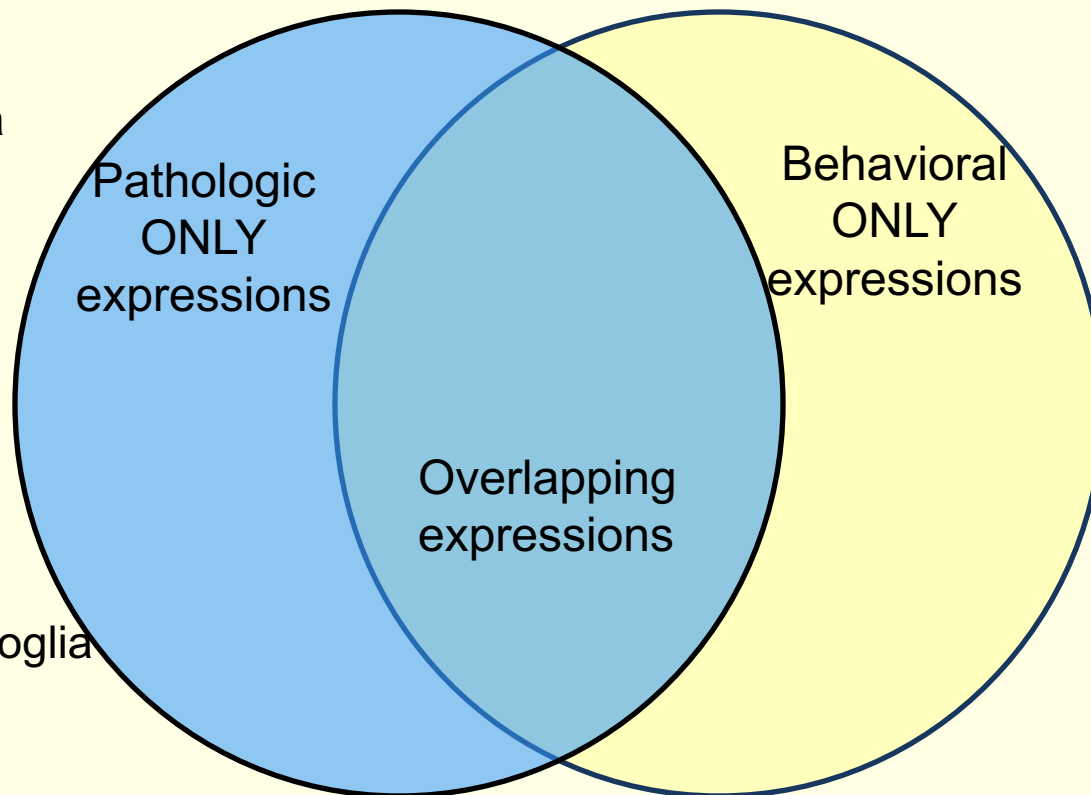
- ▶ What spectrum of neurotoxicity is appropriate for a safety screen?
- ▶ Behavioral or pathologic evaluation: is there a choice which to use?
- ▶ Any change/injury could be recoverable or permanent-how to distinguish which?
- ▶ The brain can be evaluated using thousands of end points. It is not reasonable to assess all of them.

***Luckily, there are definitive, relatively simple solutions to these challenges***

# Behavioral and pathological assessments are complementary approaches

## Pathologic

Reactive microglia  
Reactive astrocytes  
Alterations in Neurotransmitters  
Changes in gene expression  
Death of neurons, Astrocytes or microglia



## Behavioral

Motor incoordination  
Sensory deficits  
Altered states of arousal  
Learning and memory impairment  
Neurological dysfunction such as seizure, paralysis, tremor

***Each approach has its strengths and challenges. Each is necessary and uniquely capable of detecting specific expressions of neurotoxicity***



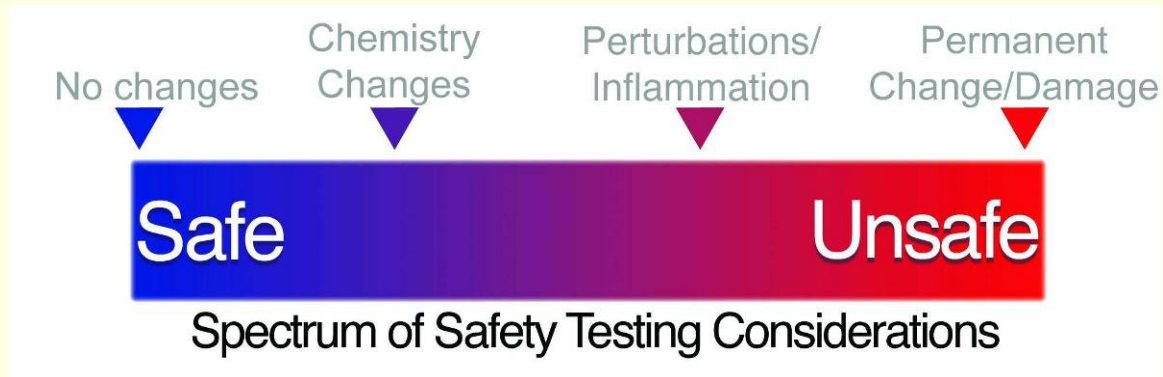
# What defines neurotoxicity?

- ▶ Loosely: “Anything that represents a departure from normal in the CNS”
- ▶ For pharmaceuticals, especially neuroactive compounds, change from normal is the objective.
- ▶ How then to determine Negative Changes and what qualifies as a safety risk?



*Today's scope will be limited to the detection of Negative Changes*

# Spectrum of Pathologic Endpoints

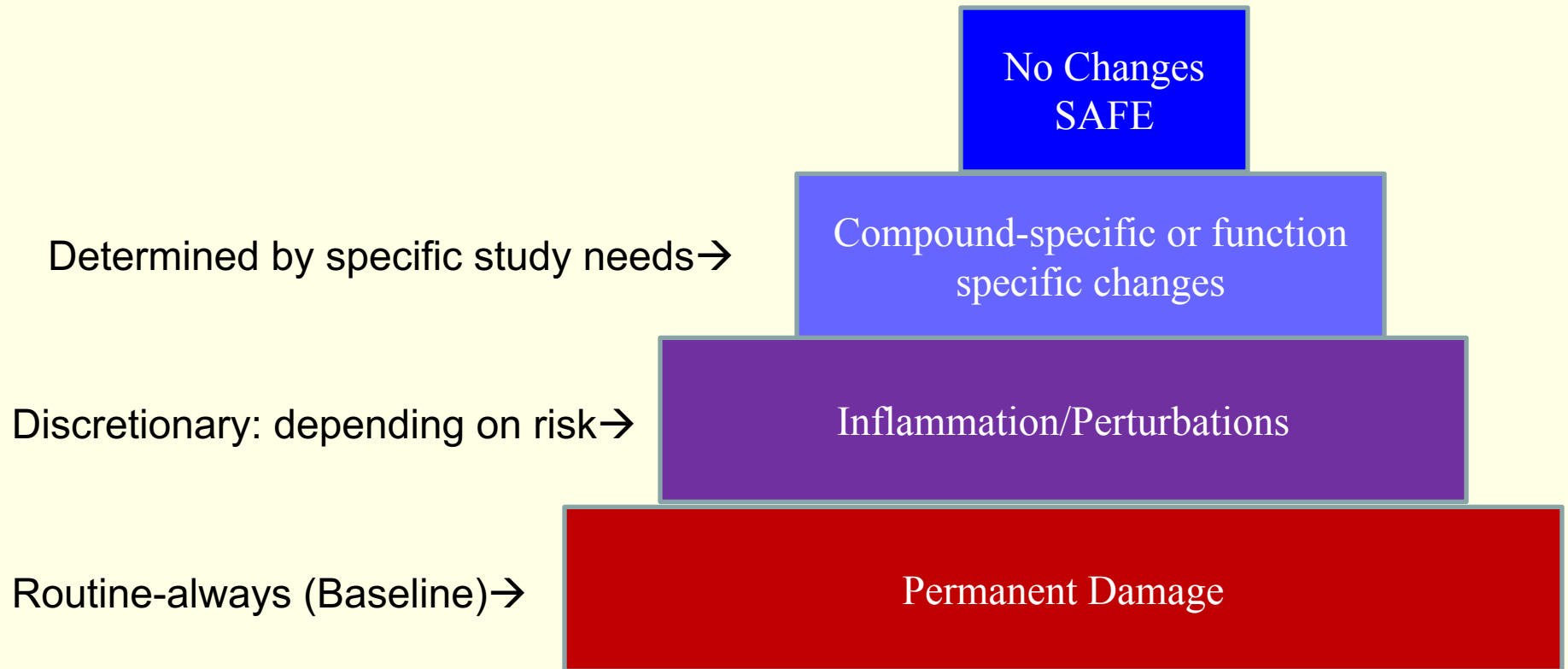


***There can be a standard level of risk enforced as acceptable by the FDA or this can be subjective based on a treatment and risk/benefit factors***

## Goals of this discussion

- ▶ Focus on identification of baseline testing, modifier's to the baseline and increments to the baseline
- ▶ The full spectrum of possible endpoints will be discussed
- ▶ Provide the endpoints, rationale to achieve them and specific guidance on protocol designs to accommodate a host of variables

# Stepwise Approach to Testing



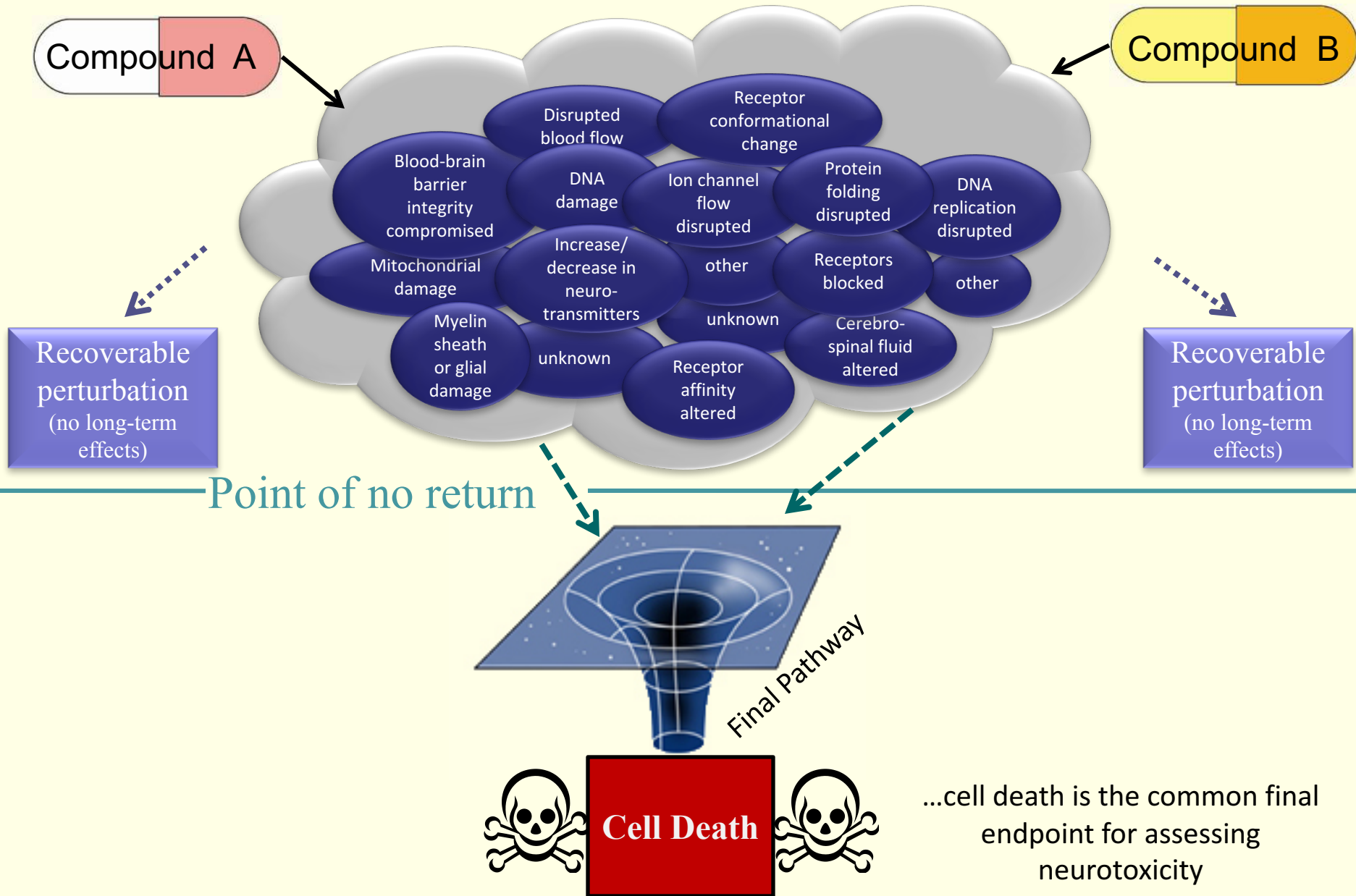
***Studies can be customized depending on risk/benefit considerations BUT the most basic safety risks should always be assessed***

# The brain has the potential to mask the difference between recovery and compensation

- ▶ Following permanent damage or injury, the brain can seemingly function “normally”
  - ▶ With a recoverable injury, the brain actually returns to “health” and there is no permanent implication
  - ▶ Following permanent damage, the brain is often resilient and able to functionally compensate for permanent injury
- ▶ Whether compensation occurs or not, any permanent damage is significant
  - ▶ Compensation may mask the functional significance of damage
  - ▶ The brain is less capable of compensating for future insults

***Safety assessments can distinguish permanent injury from a reversible perturbation***

# Potential neurotoxins can set off a cascade of events



# Detection of Changes:

## What stain to use for safety assessment?

H&E	GFAP
Amino CuAg	Iba1
CuAg	CD68
FluoroJade B	Caspase-3
FluoroJade C	Caspase-9
Thionine Nissl	TUNEL
ChAT	NeuN
	....

***The stain is just a tool designed for a specific purpose. The correct first question to ask is “what am I trying to detect?”***

# Common stains and their specific endpoints

	Degeneration				Perturbation/ Inflammation	Other
STAIN	Neuron	Axons	Dendrite	Terminal		Custom
FluoroJade	X	X	X	X		
CuAg Methods	X	X	X	X		
H&E	X					
Nissl	X					
TUNEL	X					
Caspase	Apoptosis					
GFAP					Astrocytes	
Iba1					Microglia	
ChAT						Cholinergic
Stem Cells						Detection and proliferation

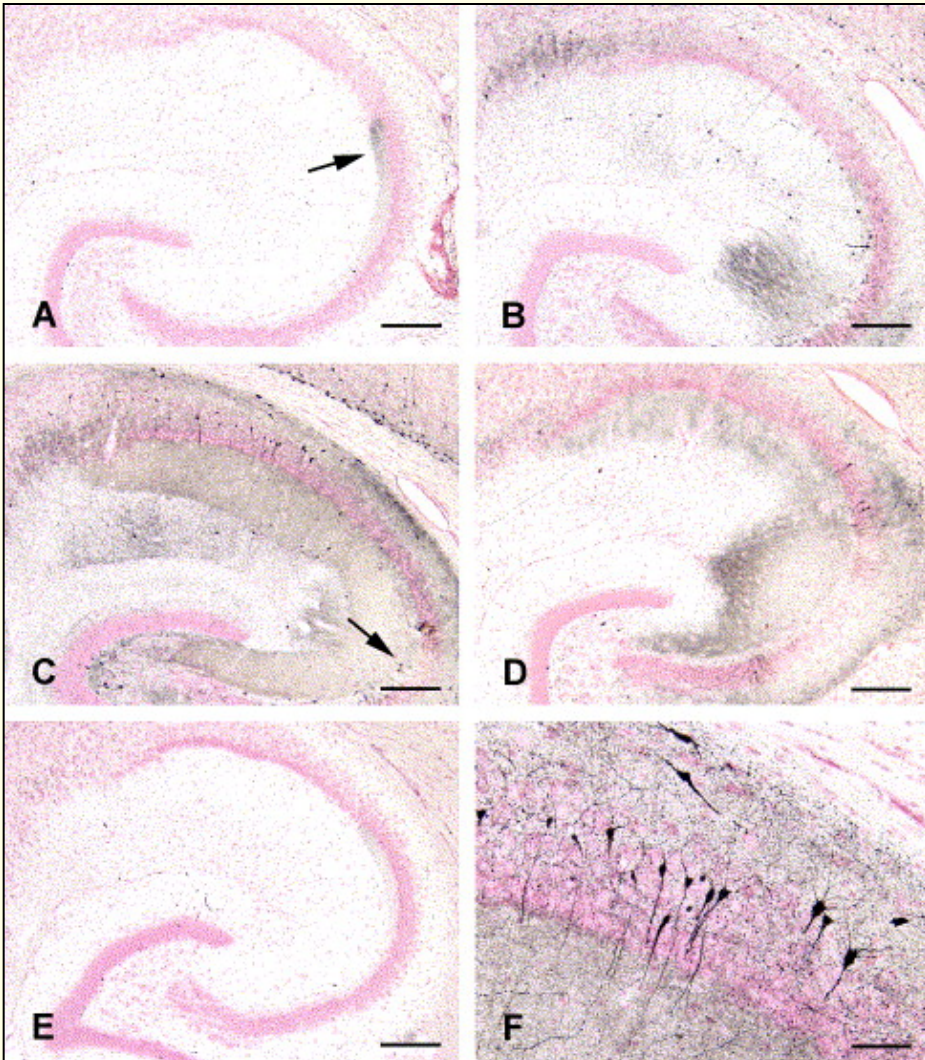
*There is no single best stain, rather stains are function-specific depending on defined endpoint to be detected. More details to follow...*



# Quick profile of common stains used in neurotoxicity testing

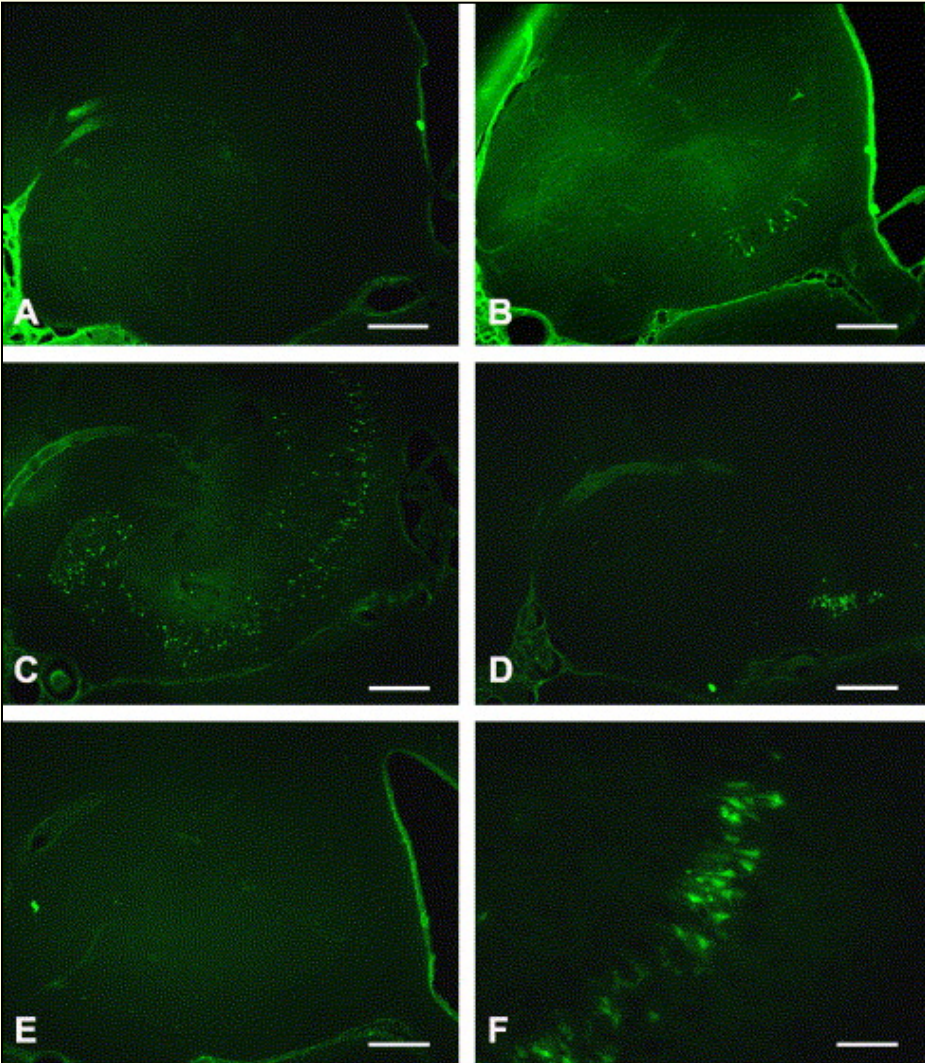
# Degeneration Stain: CuAg and Amino CuAg methods

- ▶ Light microscopy method
- ▶ Capable of staining all neuronal elements
- ▶ Only stains a positive signal for degeneration



# Degeneration Stain: FluoroJade Staining

- ▶ Fluorescent microscopy marker
- ▶ Capable of staining all neuronal elements
- ▶ Only stains a positive signal for degeneration

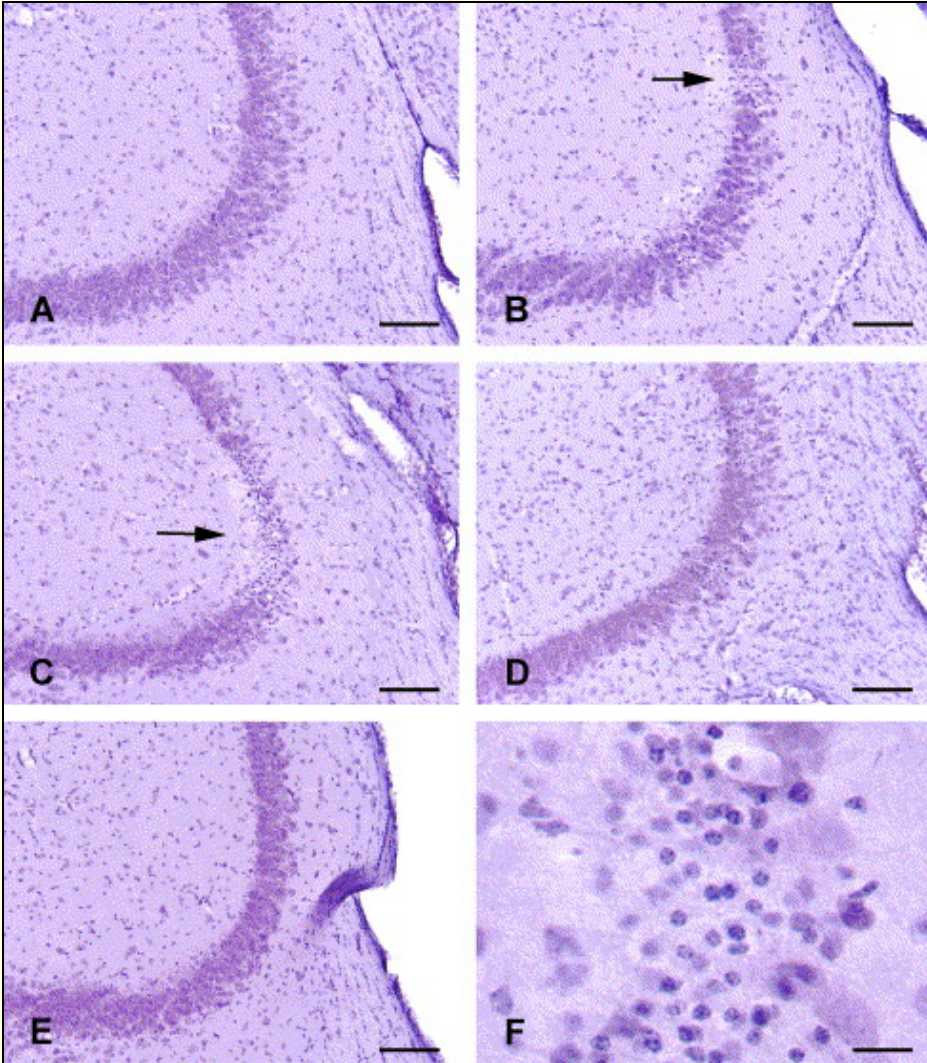




# H&E

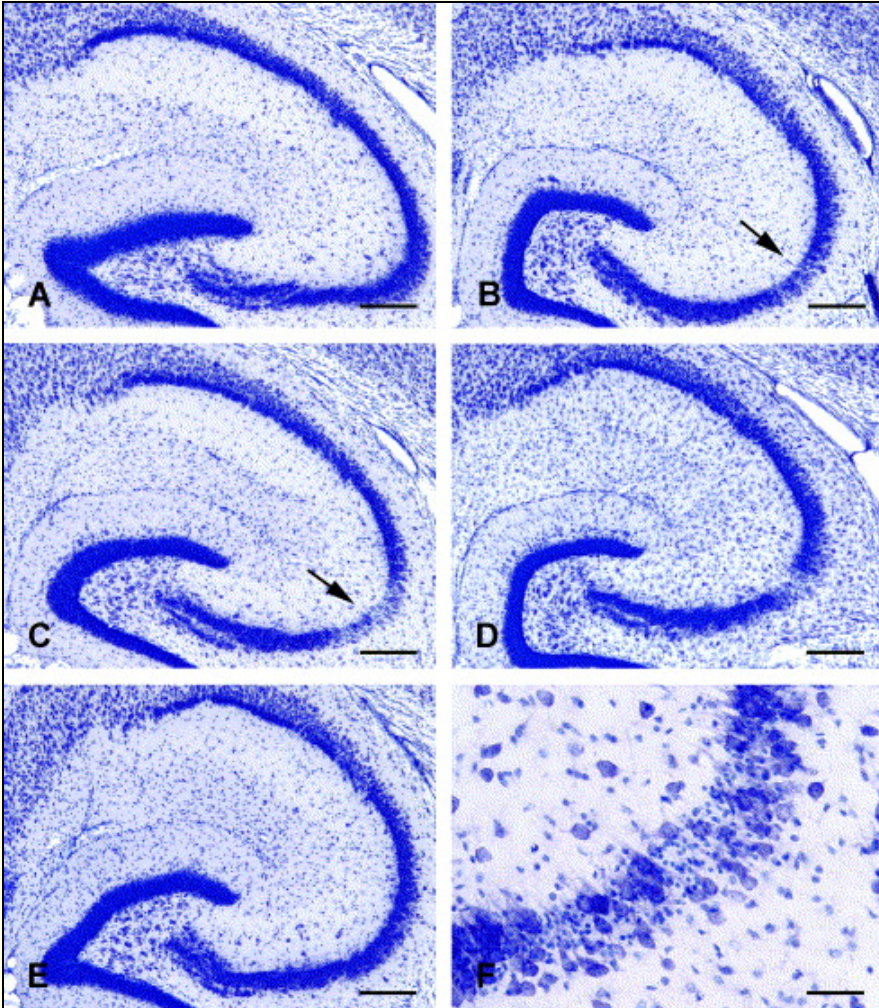
## (hematoxylin and eosin)

- ▶ Stains for cell body morphology
  - ▶ Hematoxylin stains basophilic structures (*e.g.*, cell nucleus) blue-purple
  - ▶ Eosin stains eosinophilic structures (*e.g.*, cytoplasm) bright pink
- ▶ Specialty stain for cell bodies
- ▶ Does not stain axons, terminals or dendrites



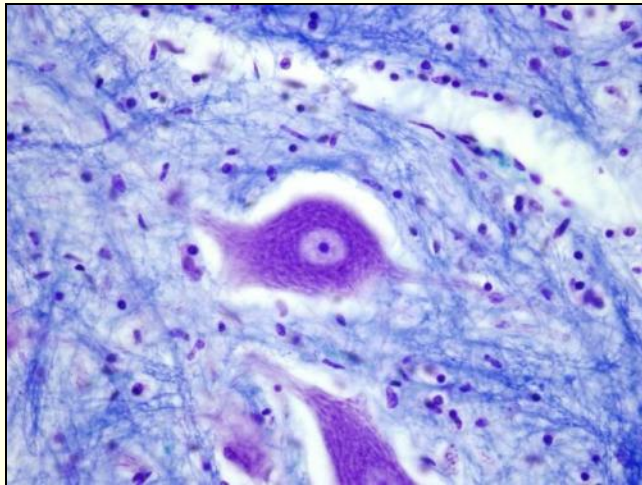
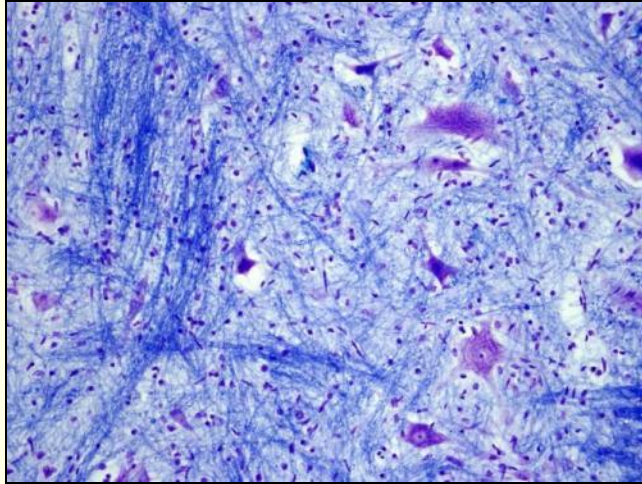
# Nissl Staining

- ▶ Stains for RNA. Uses basic aniline to stain RNA blue.
- ▶ Specialty stain for cell bodies
- ▶ Does not stain axons, terminals or dendrites





# Luxol Fast Blue

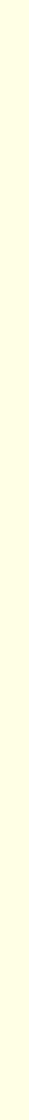


- ▶ The stain works via an acid-base reaction with the base of the lipoprotein in myelin.
- ▶ Myelinated fibers appear blue. Counterstaining with a nissl stain reveals nerve cells in purple. (e.g. cresyl violet)
- ▶ Stains myelin.
- ▶ Does not stain axoplasm, cell bodies, terminals, or dendrites.

# TUNEL method

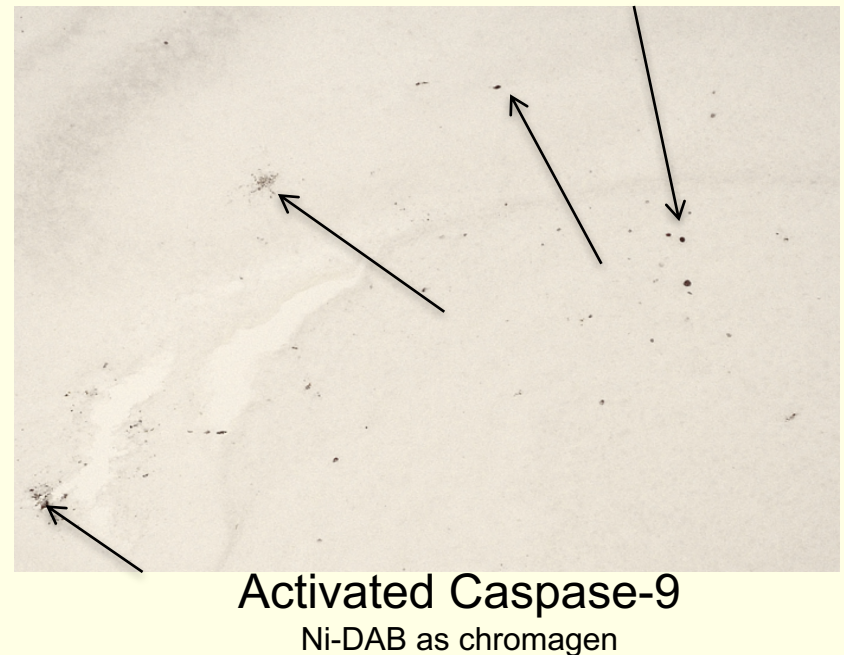
## (Terminal transferase dUTP nick end labeling)

- ▶ Stains for DNA fragmentation
  - ▶ Identifies nicks in the DNA by staining terminal transferase, an enzyme that will catalyze the addition of dUTPs that are secondarily labeled with a marker
- ▶ Specialty stain for cell bodies (nucleus of the cell)
- ▶ Does not stain axons, terminals or dendrites



# Activated Caspase-3 and Caspase-9 (species dependent)

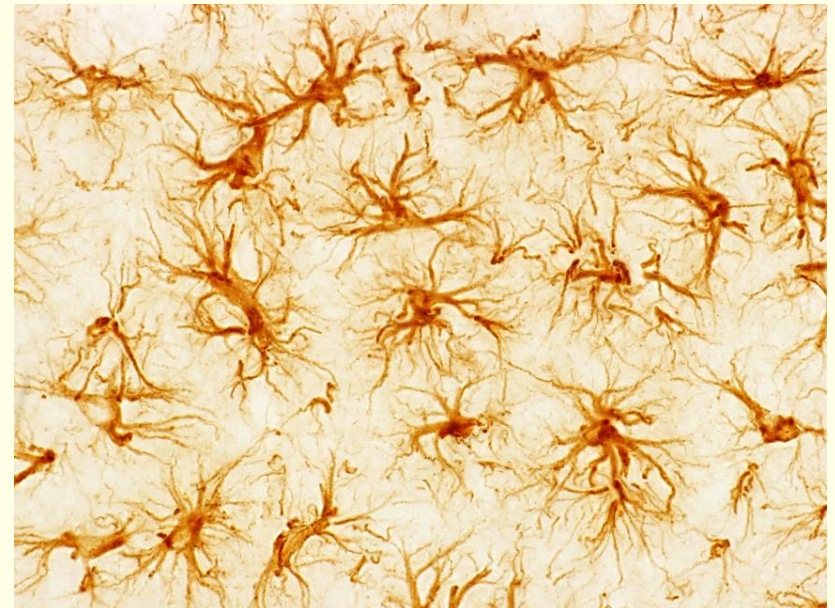
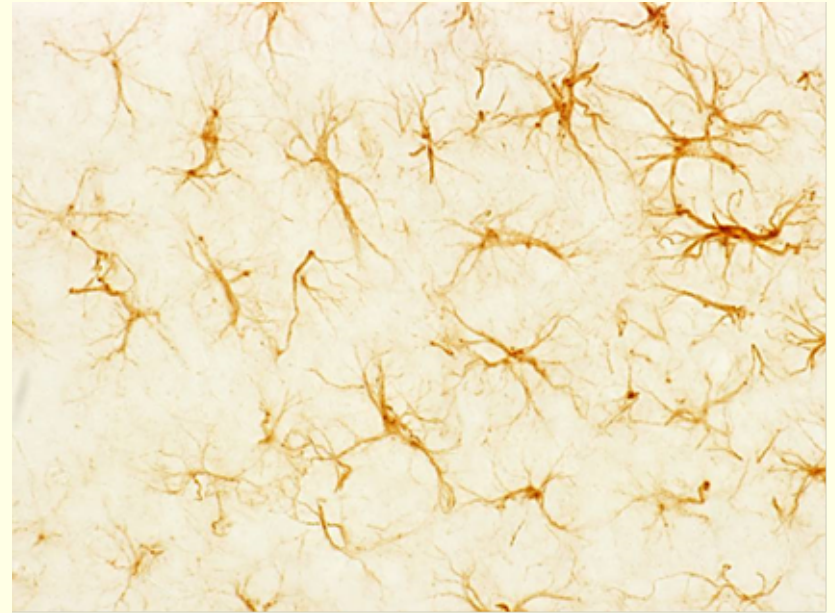
- ▶ Precursor marker for apoptosis: cell will later disintegrate
- ▶ Detectable earlier than cell disintegration and for a shorter window of time
- ▶ Caspase-3 works well in mouse and Caspase-9 works well in rat





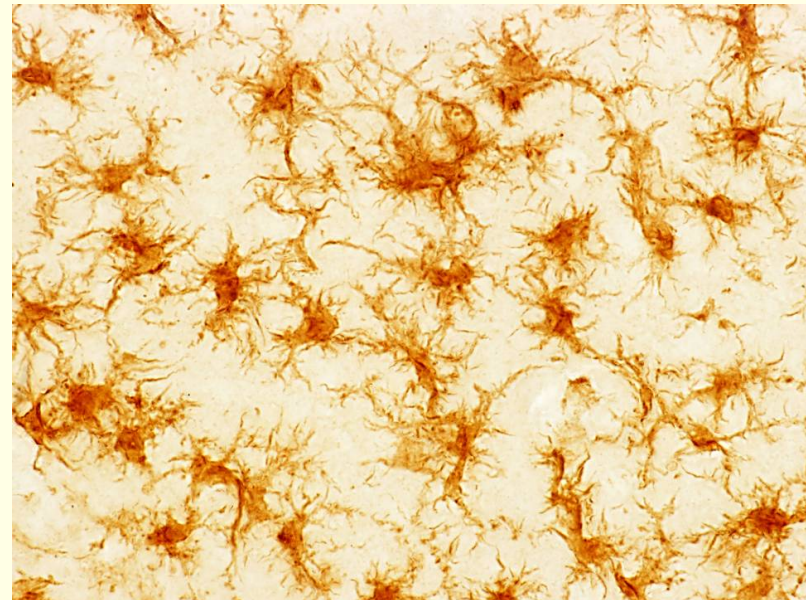
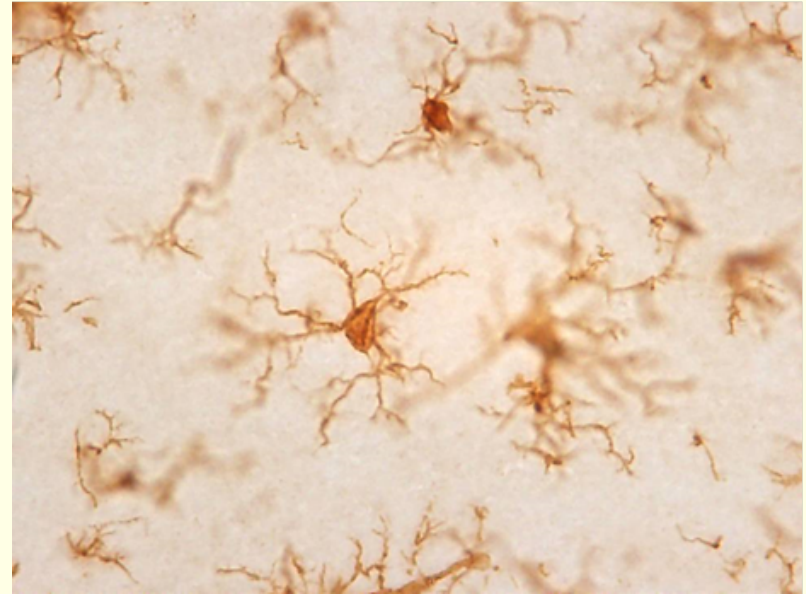
# GFAP

- ▶ Normal morphology of astrocytes revealed by IHC with an antibody against glial fibrillar acid protein (GFAP) a cytoskeletal protein unique to astrocytes.
- ▶ Reactive astrocytes displaying more numerous and thickened processes, and enhanced density of staining.
- ▶ Changes in the attributes of GFAP staining are most noticeable beginning 36-48 hours following an insult, peaking at ~ 72 hours and persisting in potentially diminishing fashion for weeks (and sometimes much longer)



# Iba1

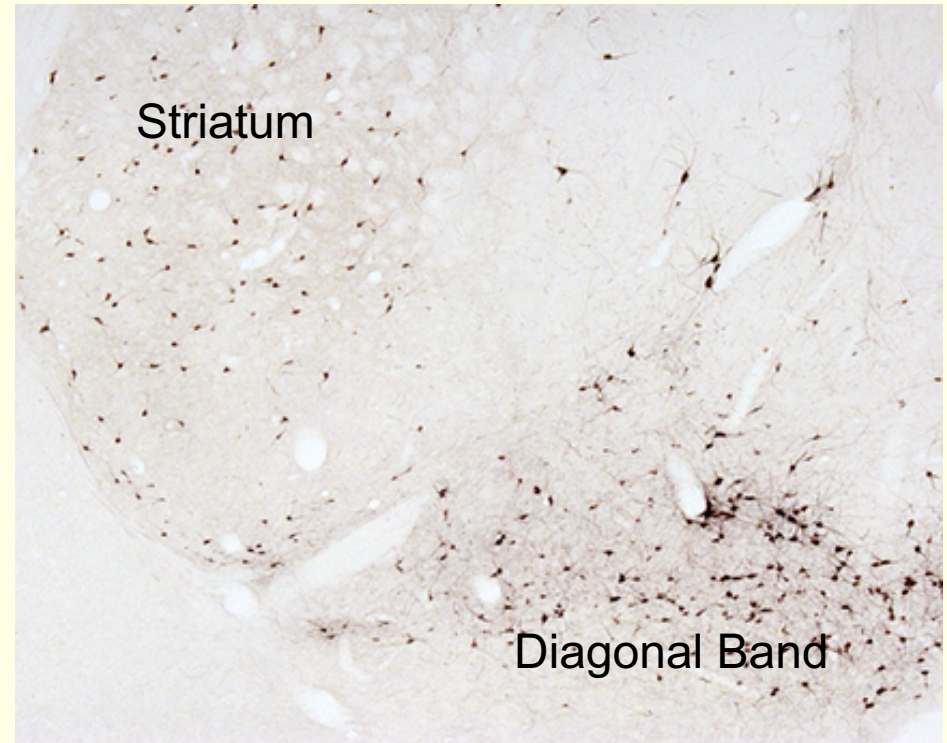
- ▶ Reactive microglia responding to perturbations display a hypertrophy shown by IHC with Iba1 antibody.
- ▶ The cell body becomes enlarged, processes become fewer until there may be none (amoebiform state), and there is also a tendency to cluster into ‘knots’
- ▶ Iba1 IHC reveals the most activity beginning ~4-5 days after an insult or exposure, peaking at 5-7 days and persisting for two weeks or more.
- ▶ CD68 also reveals a subset of activated microglia





# Choline Acetyl Transferase (ChAT)

- ▶ Drugs affecting Nerve Growth Factor (NGF) can affect the cholinergic cell population.
- ▶ Analysis of the status of the cholinergic population utilizes the enzyme involved with the synthesis of acetylcholine, Choline Acetyl Transferase (ChAT).
- ▶ ChAT positive neurons are widespread in the brain, but the target for analysis of for a number of would be drugs affecting the cholinergic system has been the nucleus of the diagonal band of Broca and Meynert's nucleus basal is, both found in the ventral forebrain. Both structures project heavily to the neocortex. Meynert's nucleus has been found to be depleted in Alzheimer's disease.



## Stains as basis for study design?

Stains are merely tools with very specific functions. Before selecting a stain, it is imperative to decide what to look for and how to design the study to allow the stain to reveal that underlying pathology

# Defining Baseline Testing Requirements

- ▶ The destruction of brain cells by a single acute dose is the most overt expression of neurotoxicity and easy to assess
  - ▶ Destruction of neuronal cells is the worst case scenario
  - ▶ There is no recovery from cell death
  - ▶ Cell death is the hallmark profile of unrecoverable events in the brain
  - ▶ Pathologic detection of cell death is definitive for neurotoxicity

***Baseline testing will include acute cell death assessment at a minimum***

Principles involved in the assessment of acute cell death are relevant to all other assessments along the safety spectrum.

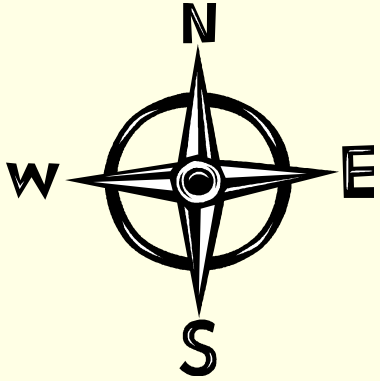
The next section will focus on the design considerations for acute cell death in great detail.

Modifiers to this baseline evaluation and detection of other endpoints along the safety spectrum will follow

# ACUTE CELL DEATH EVALUATION

Core baseline NTX assessment

Permanent Damage

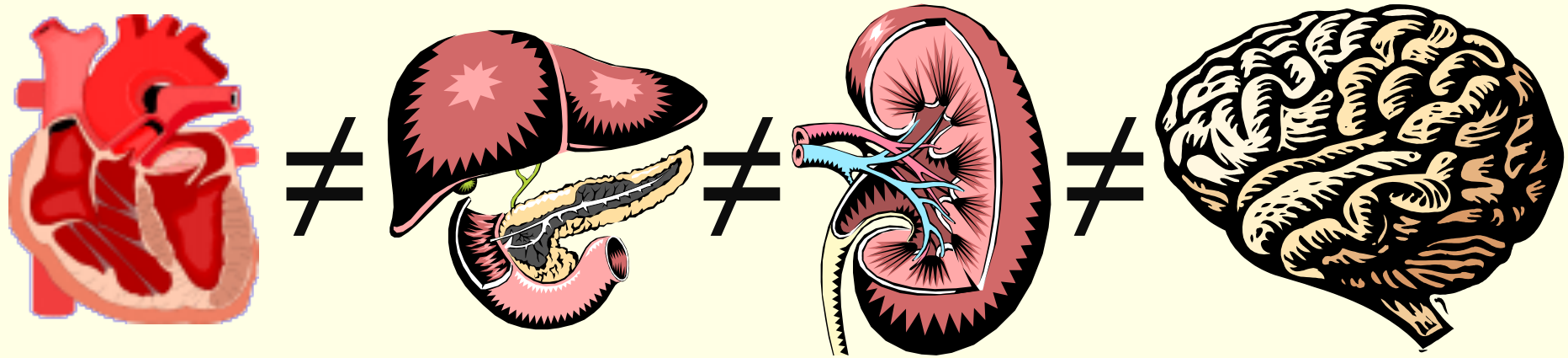


Location, location, location

A Core Principle of Neurotoxicity Assessment



Different parts of the body have unique profiles with regards to toxicity



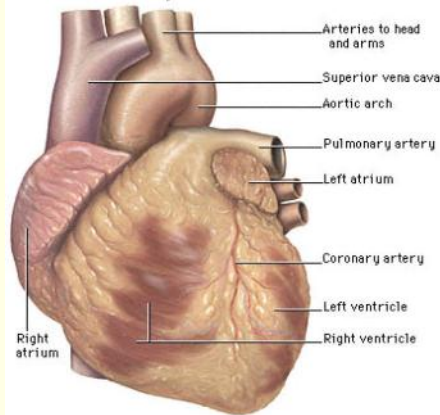
Heart ≠ Liver ≠ Kidney ≠ Brain, etc.

# Within any organ, individual anatomical elements are specifically and uniquely vulnerable to toxic agents

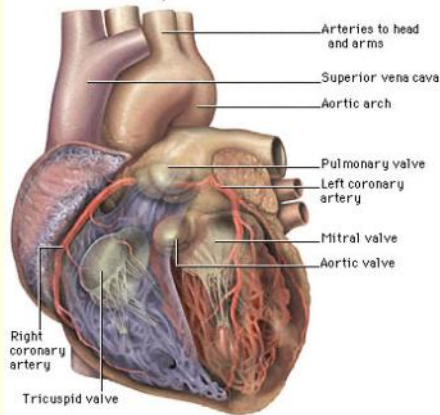
## Heart

Arteries  $\neq$  valves  $\neq$  chambers  $\neq$   
septum  $\neq$  veins  $\neq$  muscle, etc.

Exterior structures of the heart

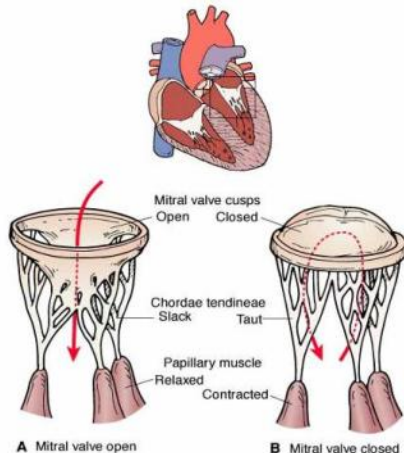


Interior structures of the heart



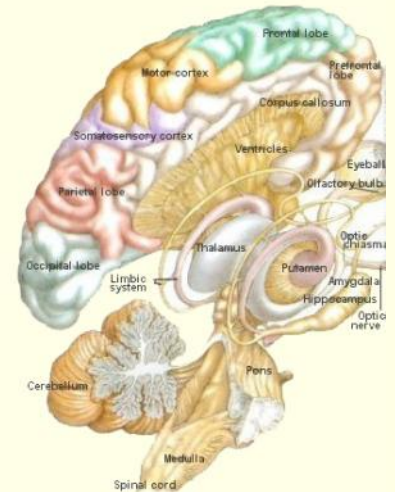
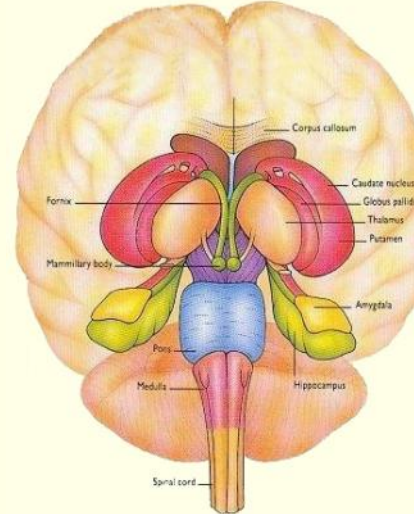
### Valves:

Aortic  $\neq$   
mitral  $\neq$   
pulmonary  $\neq$   
tricuspid



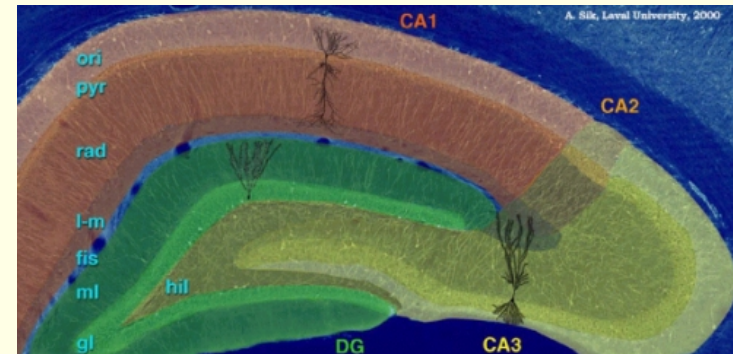
## Brain

Cortex  $\neq$  hippocampus  $\neq$  cerebellum  $\neq$   
hypothalamus  $\neq$  thalamus  $\neq$  amygdala, etc.



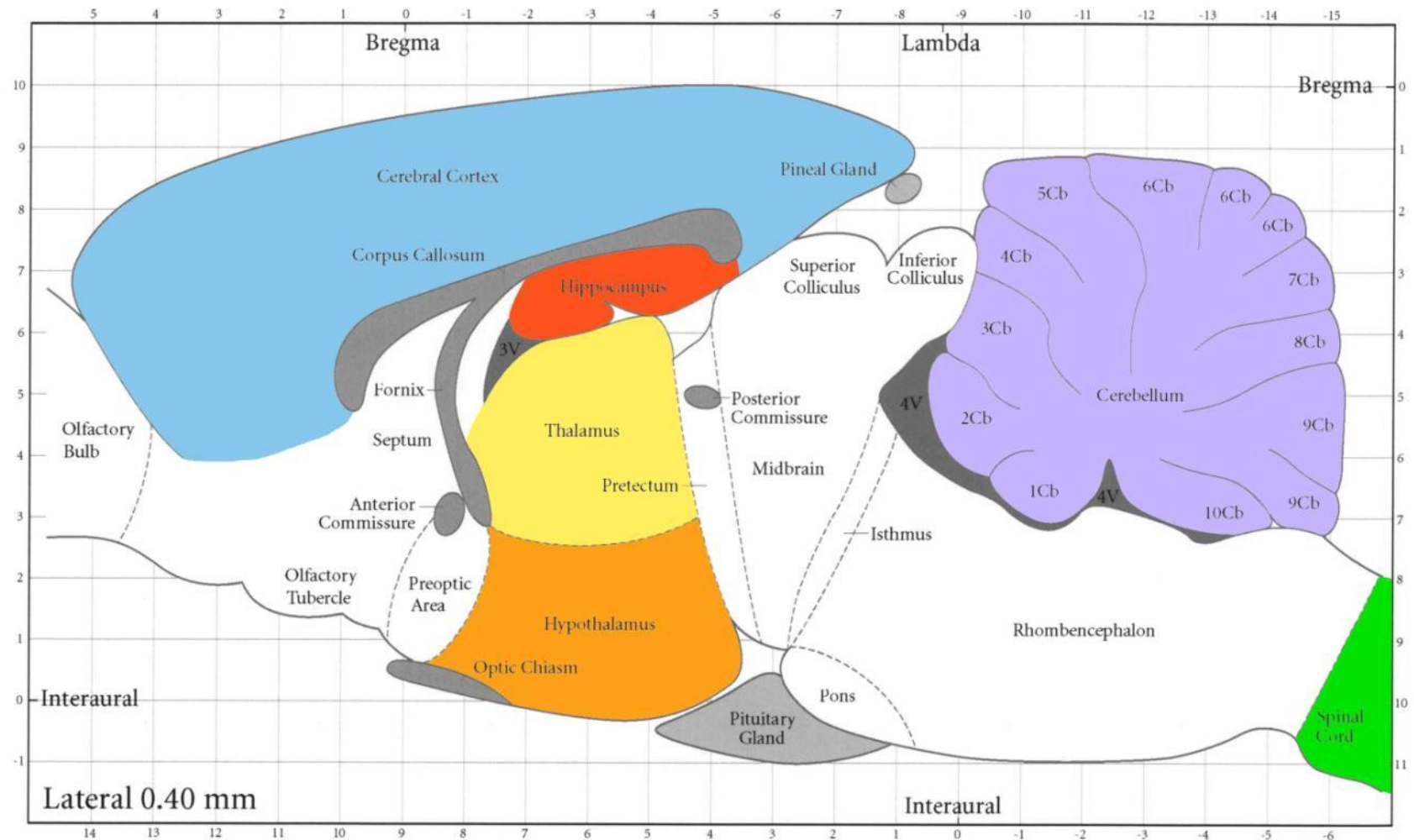
### Hippocampus:

CA1  $\neq$  CA3  $\neq$   
ventral dentate  
gyrus  $\neq$  dorsal  
dentate gyrus



***Each element warrants consideration.***

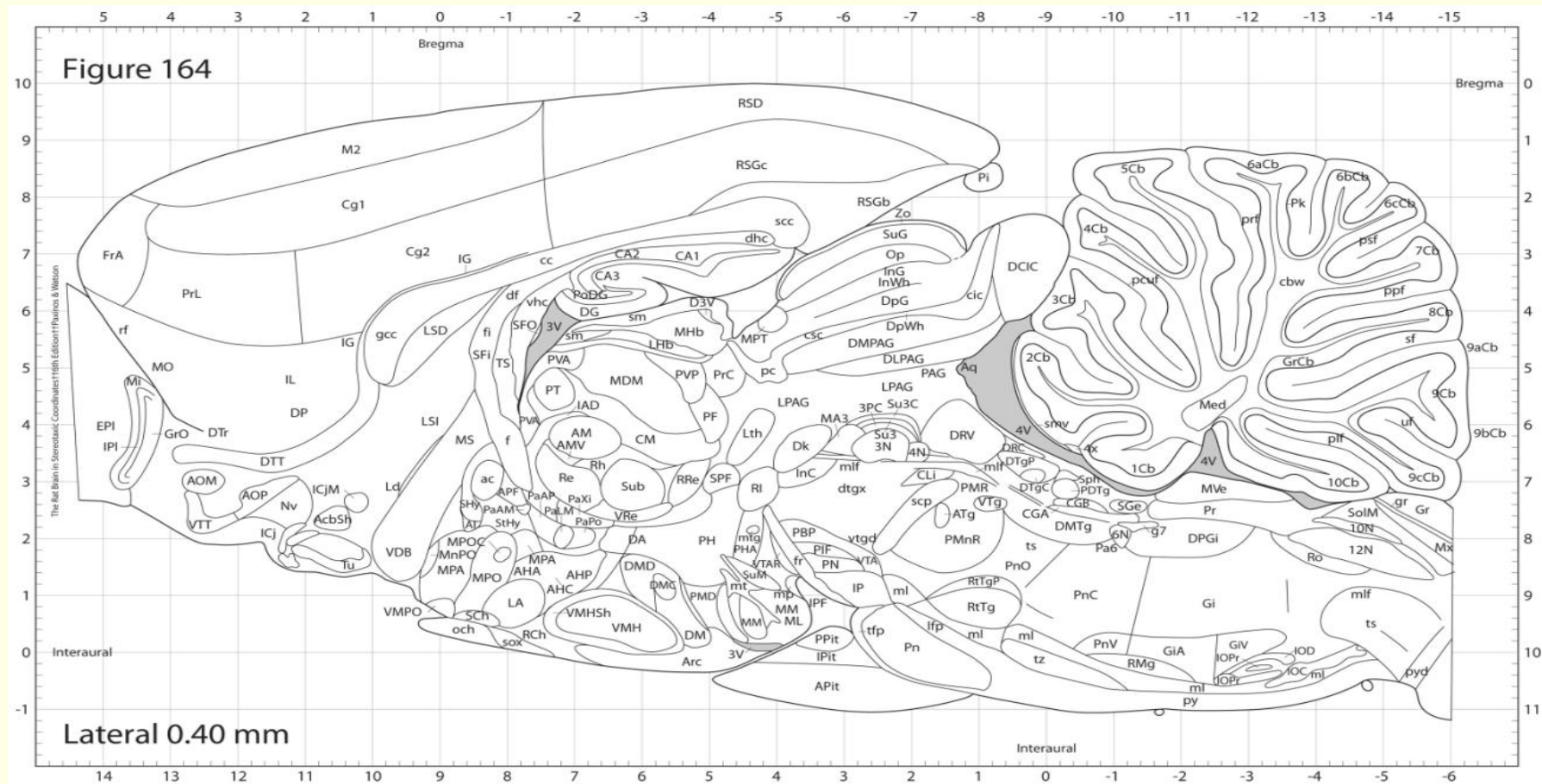
# Major divisions of the brain as represented in a sagittal plane of rat



Some major divisions are not represented in this drawing, as they are located more lateral. It is impossible to see all regions of the brain in any one section.



Each major division of the brain is comprised of many specialized populations



Most of the subpopulations of the brain are not seen in this section, as they are located more medial or lateral. It is impossible to see all regions of the brain in any one section.

Paxinos & Watson, 2007

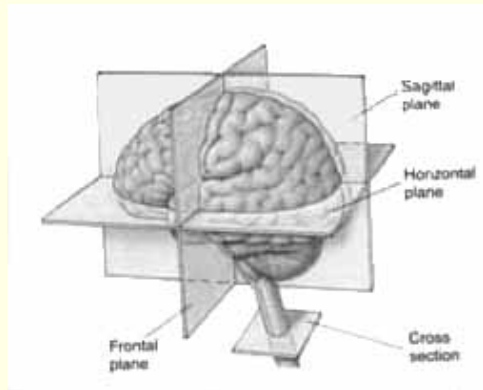
# The brain has an incredible amount of diversity and complexity

- ▶ There are over 600 distinct cell populations within the brain.
- ▶ Each division of the brain has different cell types, connectivity, and functionality.
- ▶ Brain cells in different populations of the brain exhibit unique vulnerabilities to neurotoxic compounds.
- ▶ Our understanding of the brain has been increasing exponentially but we still do not fully understand:
  - ▶ The comprehensive functions of each population
  - ▶ The interactions of all the populations
  - ▶ The symptoms or functional impact of damage to any specific population

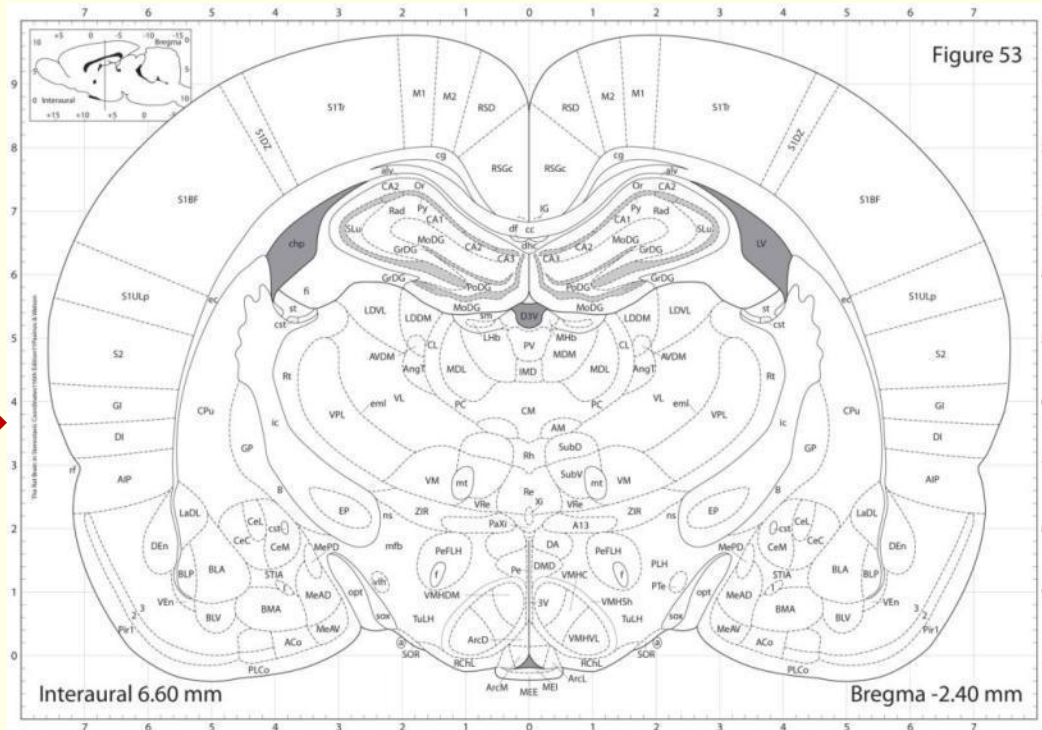
***Although our understanding of the brain is perhaps not as complete as with other organs, we don't know of any regions of non-importance. There is no appendix of the brain.***

# Illustration primer

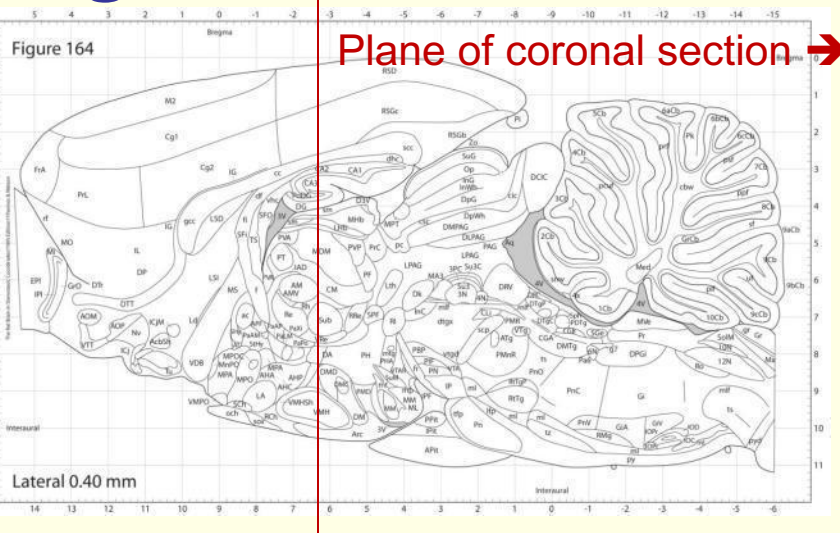
# Primer for upcoming illustrations: Planes of sectioning for analysis



## Coronal section



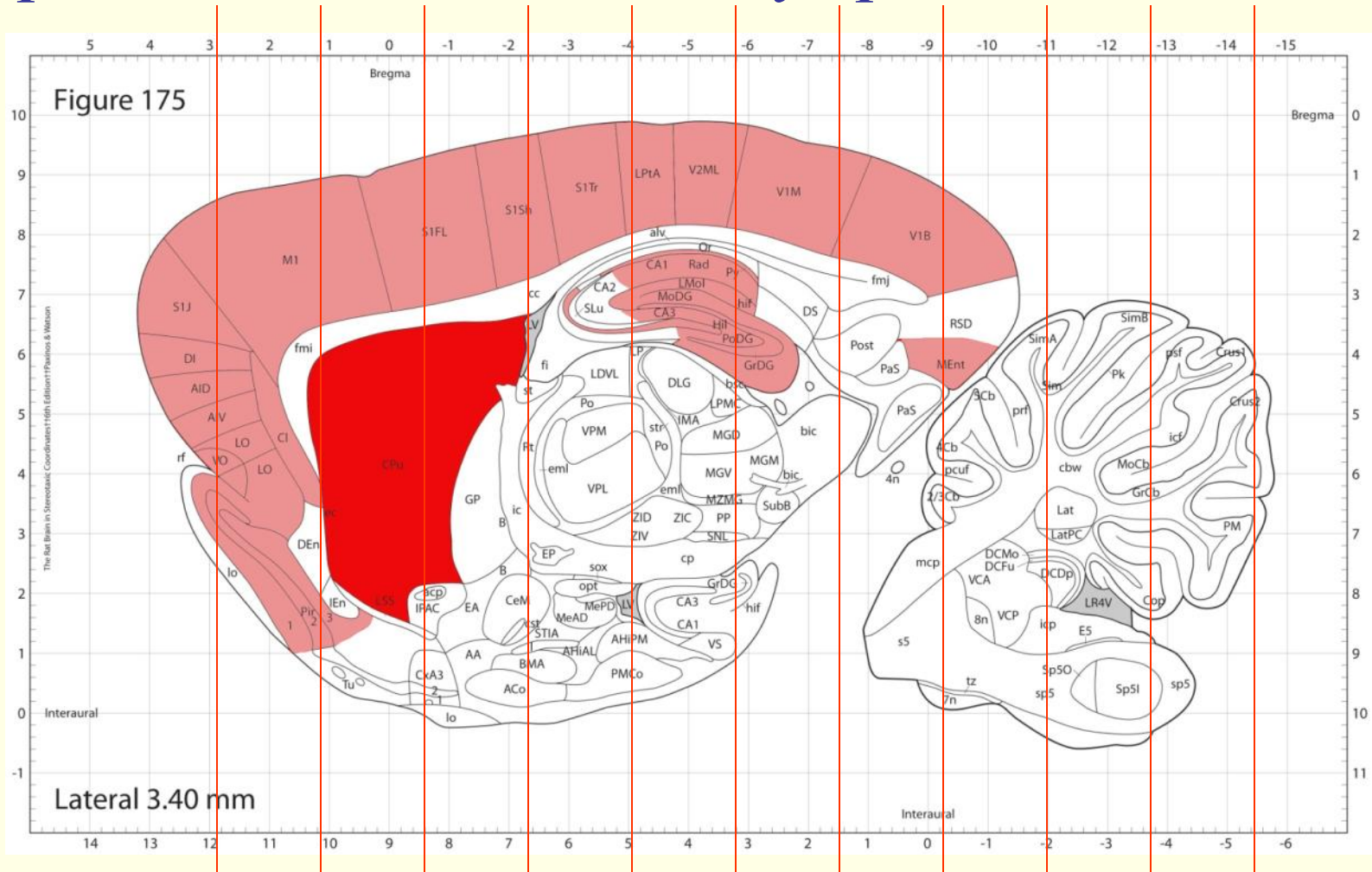
## Sagittal section



Plane of coronal section →

Any plane is suitable, however most researchers use coronal sections for analysis

From a sagittal view, we can see what affected populations are visible at any specific coronal level



### Key to Shading:



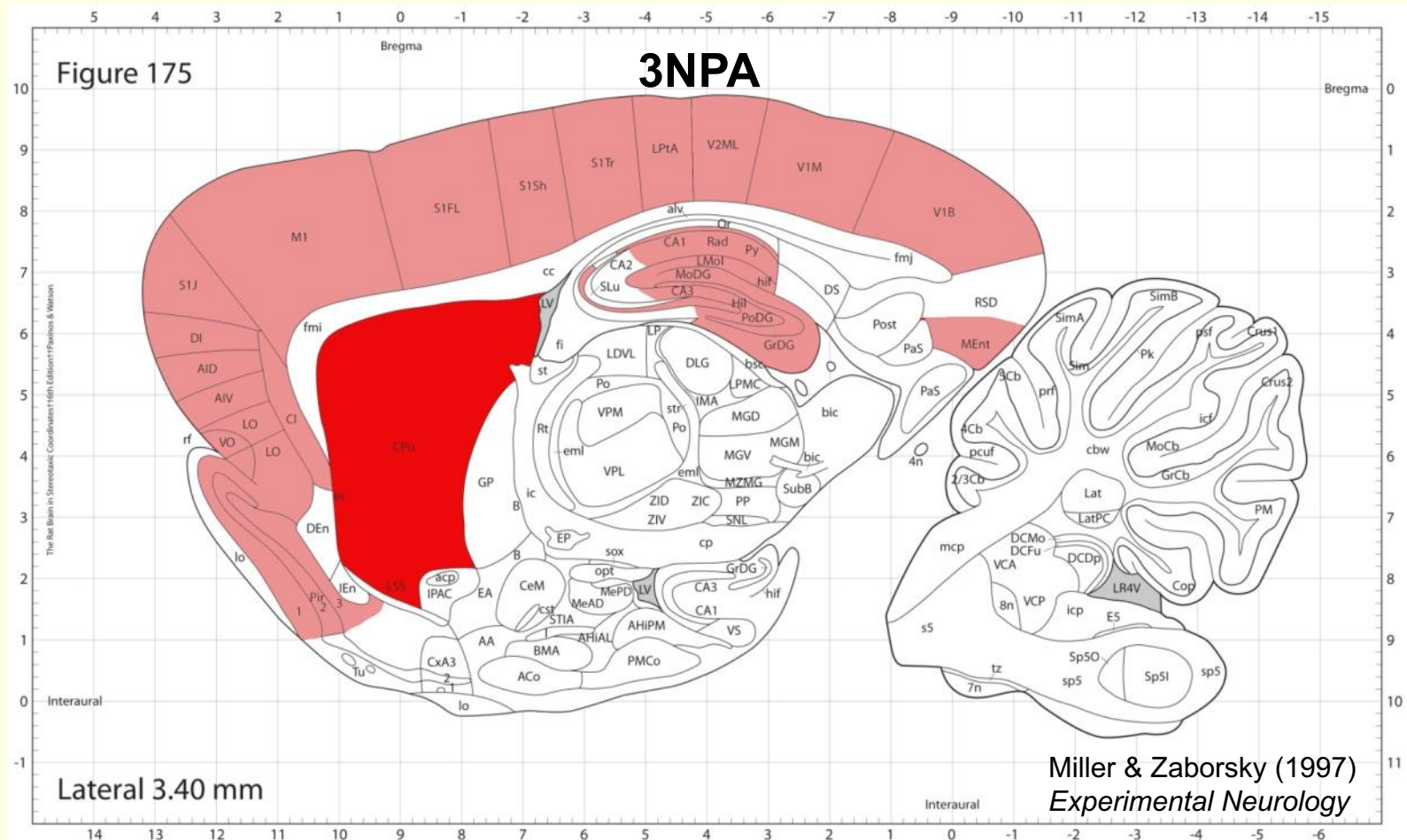
Major impacts to region  
Less pronounced impacts

The red lines represent the populations a coronal section would pass through at a particular level



Where do neurotoxins affect the brain?

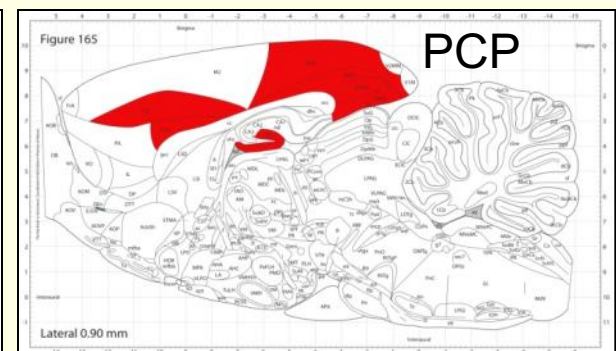
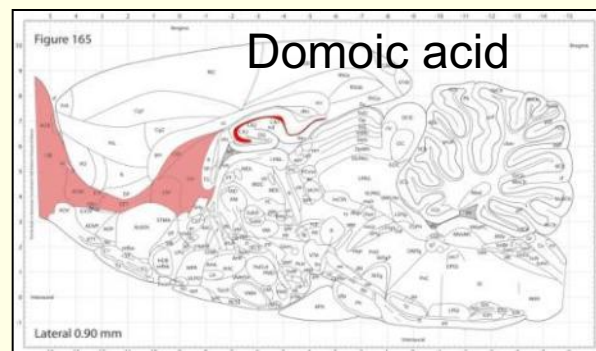
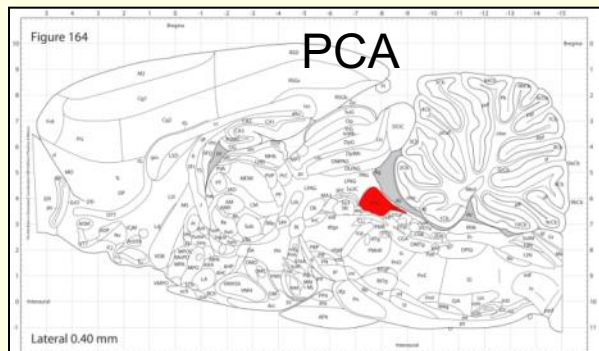
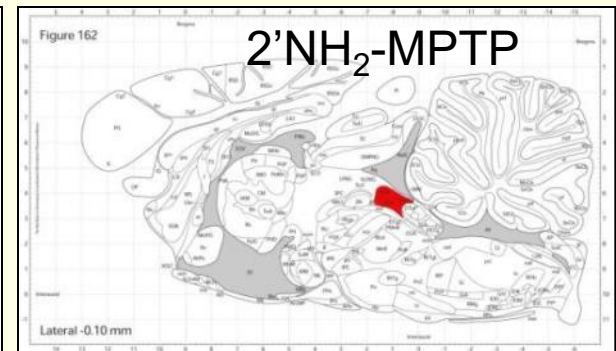
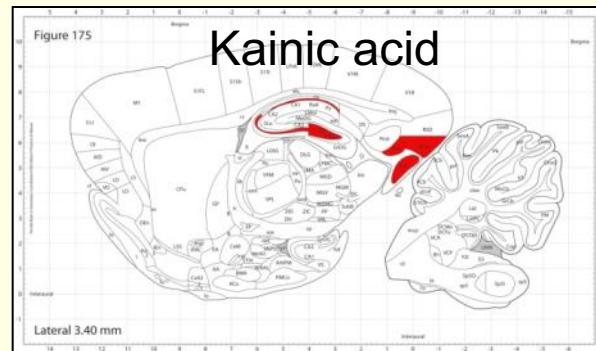
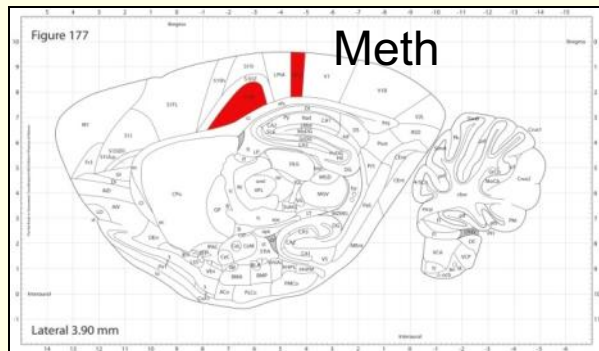
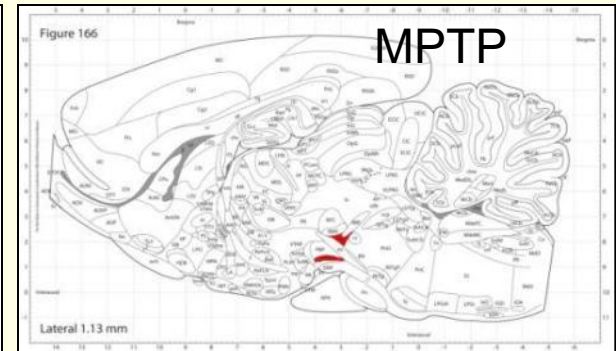
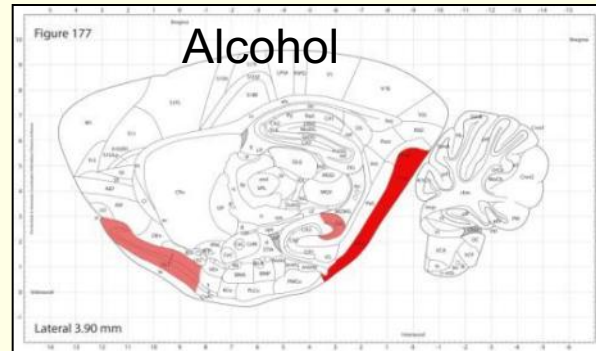
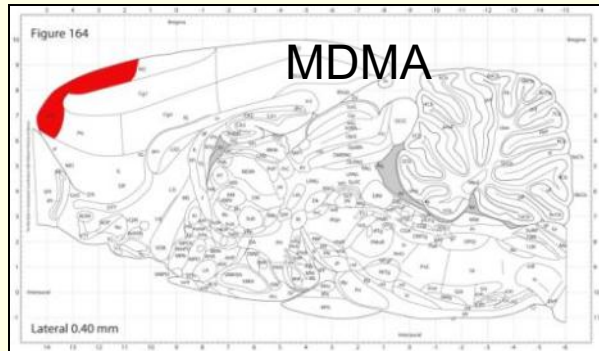
In some cases, cells impacted by a neurotoxic compound are widespread



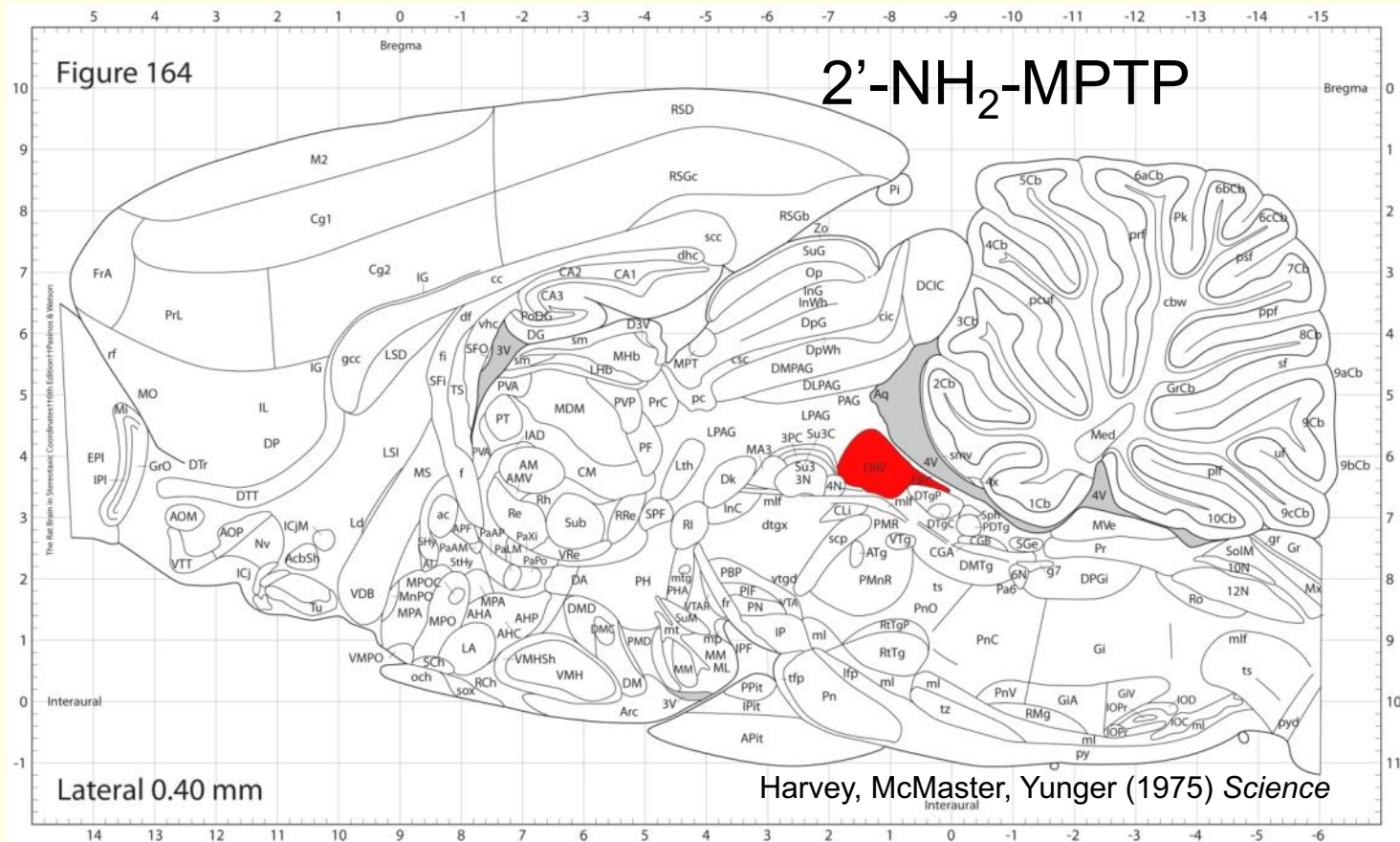
3-Nitropropionic Acid destroys cells in caudate putamen, as well as hippocampus and a number of cortical structures. 3NPA is used as an animal model for studying Huntington's Disease pathology.

*It is uncommon to have such widespread destruction*

# More often, neurotoxins kill cells in smaller portions of the brain



The volume occupied by a population of the brain does not correspond with significance



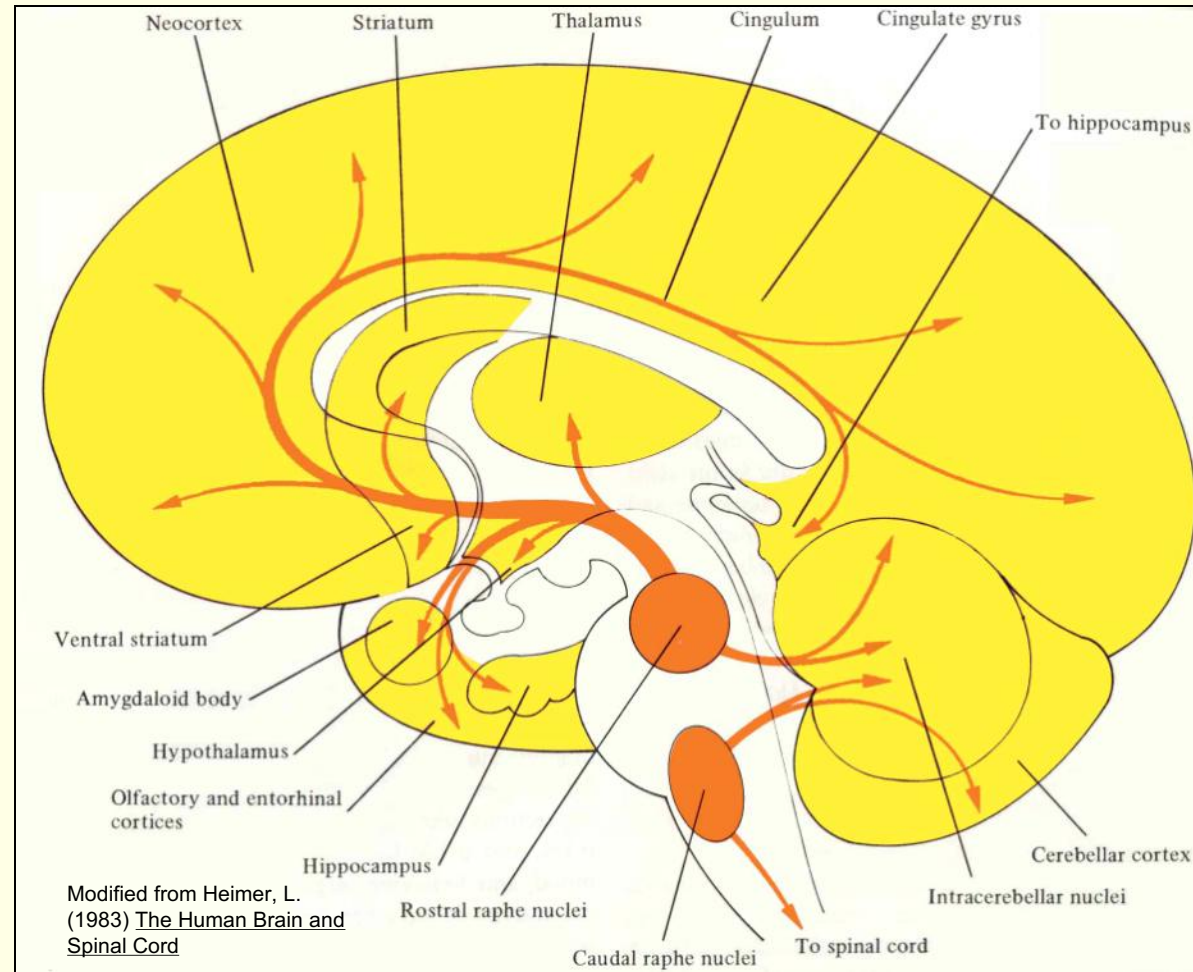
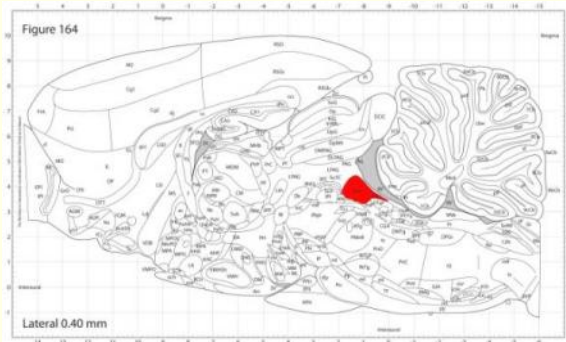
**2'-NH<sub>2</sub>-MPTP destroys cells in the raphe nuclei**

*Even the destruction of very small regions in the brain can have profound consequences*

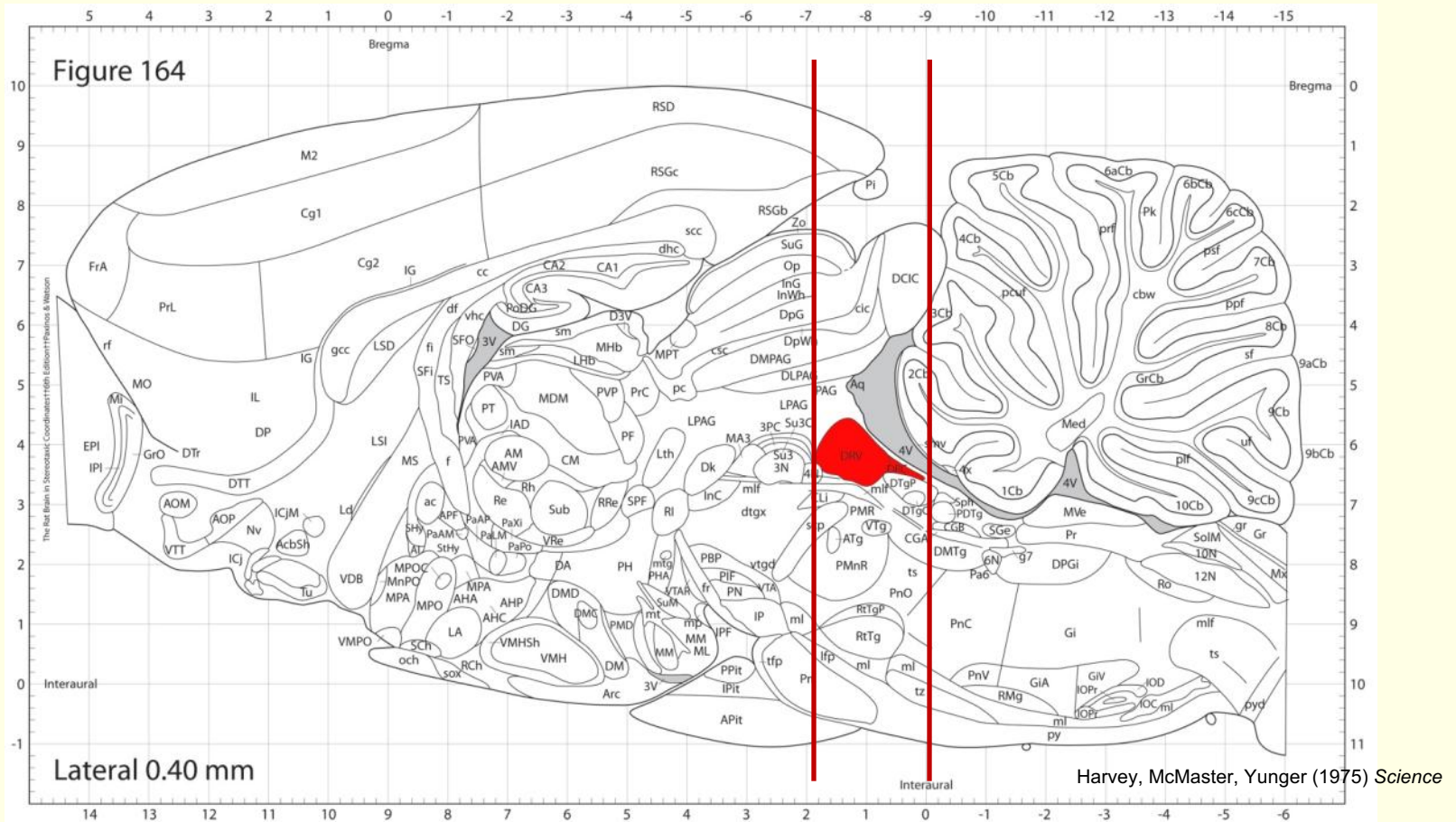


# The raphe nuclei projects serotonin throughout the brain

- ▶ Nearly all serotonergic cell bodies in the brain lie in the raphe nuclei
- ▶ Losing these cells yields profound long-term negative effects.
- ▶ Serotonin is an important neurotransmitter, involved in regulating normal functions as well as diseases (*e.g.*, depression, anxiety, stress, sleep, vomiting).
- ▶ Drugs which interact with the serotonergic system include Prozac, Zofran and many others.



While causing a large impact, the area damaged by 2'-NH<sub>2</sub>-MPTP is small and could easily be not sampled



Raphe nuclei spans less than 2mm Anterior-posterior

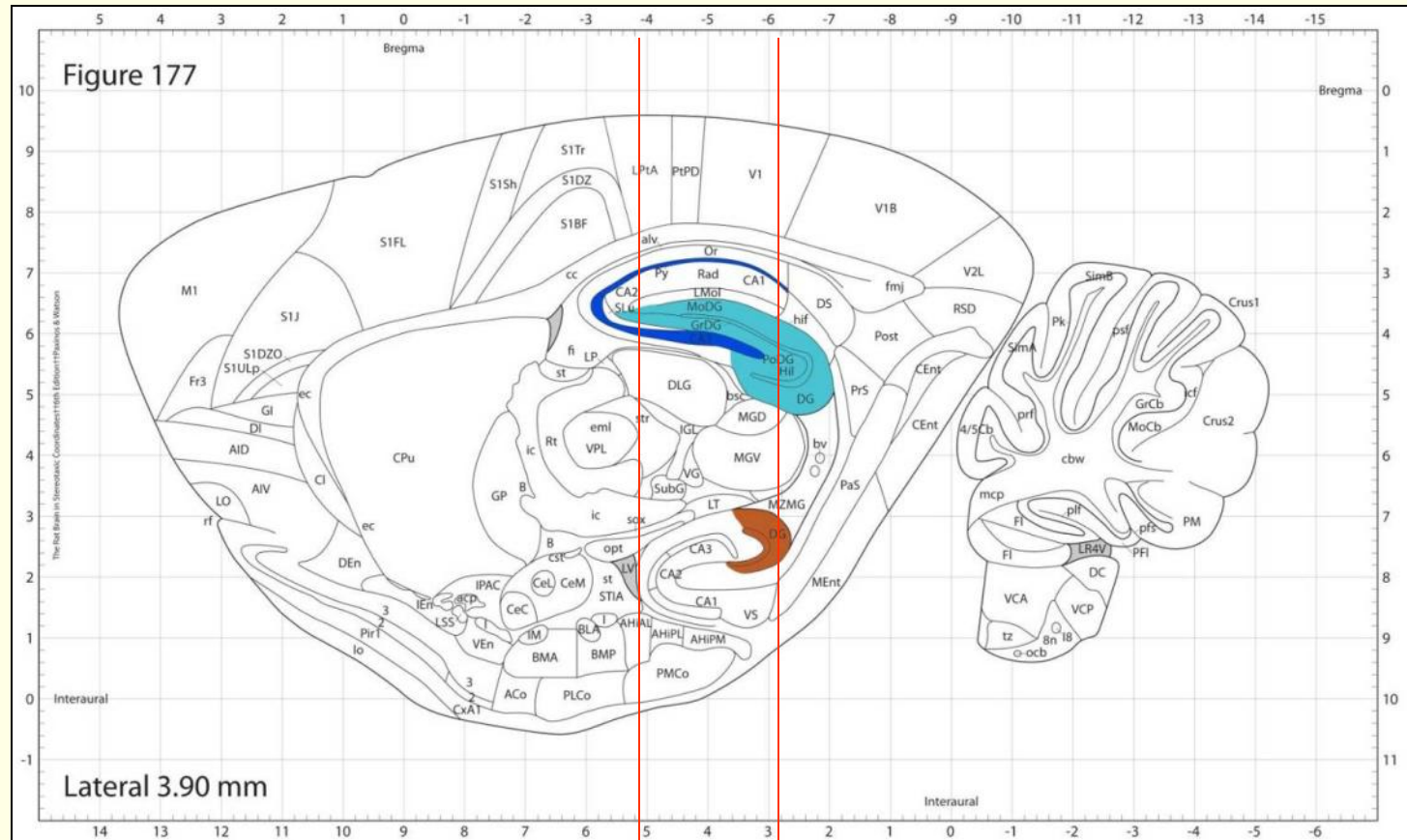
***Sampling strategies for assessment of neurotoxicity in the brain must account for small footprints of structures to be assessed***

Within the same major division, different compounds affect different subpopulations

Domoic acid  
destroys cells in  
the **pyramidal  
layer of  
hippocampus**

## PCP destroys cells in dorsal dentate gyrus

## Alcohol destroys cells in **ventral dentate formation**



(Coronal slices at these levels on the next slide)

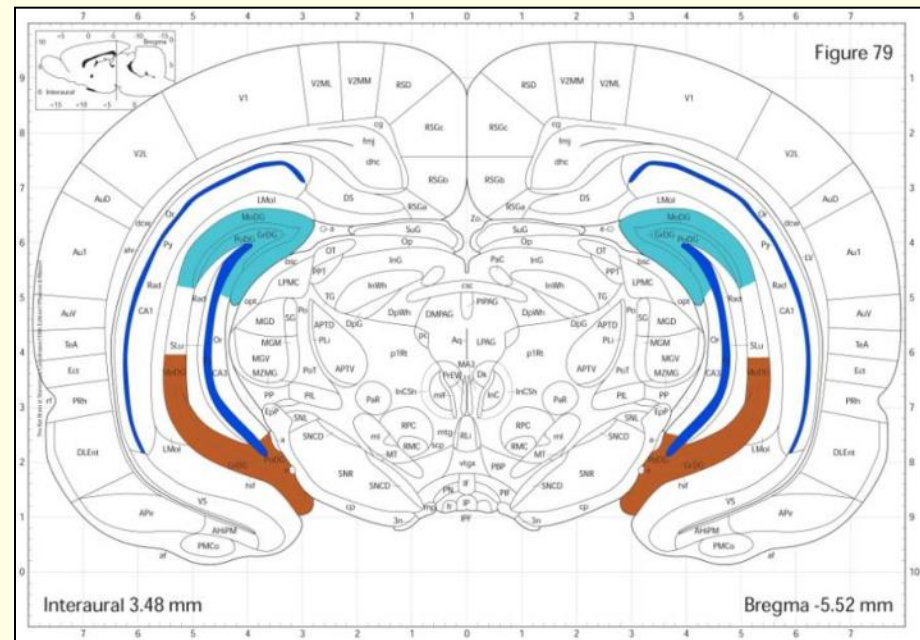
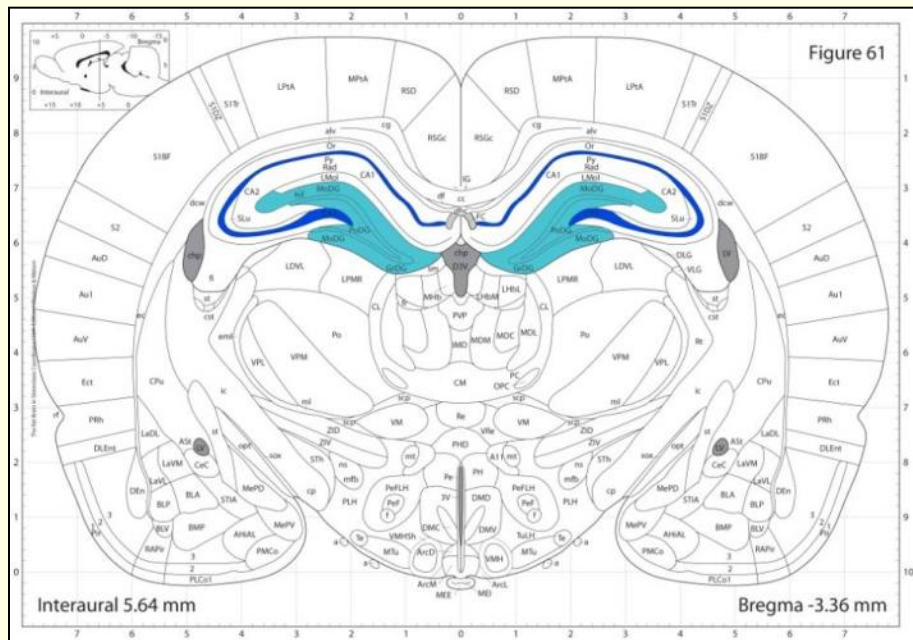
***Assessing a major division of the brain for damage requires sampling from each subpopulation of that region***



# Within the same major division, different compounds affect different subpopulations

**In a commonly used view of hippocampus, ventral structures cannot be seen**

**A more posterior section allows ventral structures to be seen**



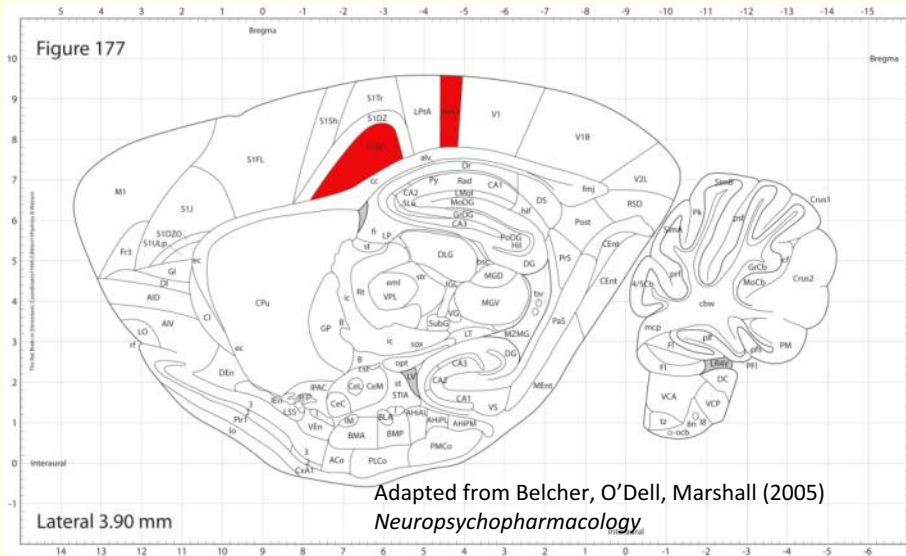
***Assessing a major division of the brain for damage requires sampling from each subpopulation of that major division***



# The location of damage in the brain is unpredictable

## Study #1:

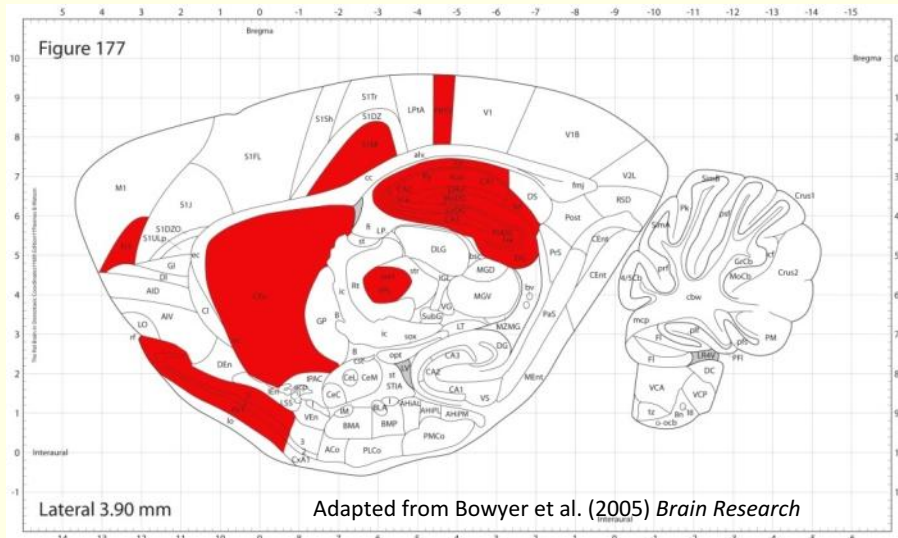
**A limited area of cell death was witnessed**



In this example, researchers anticipated, looked for and found that D-amphetamine destroys cells in parietal cortex and somatosensory barrel field cortex

## Study #2:

**Further evidence of cell death was observed**



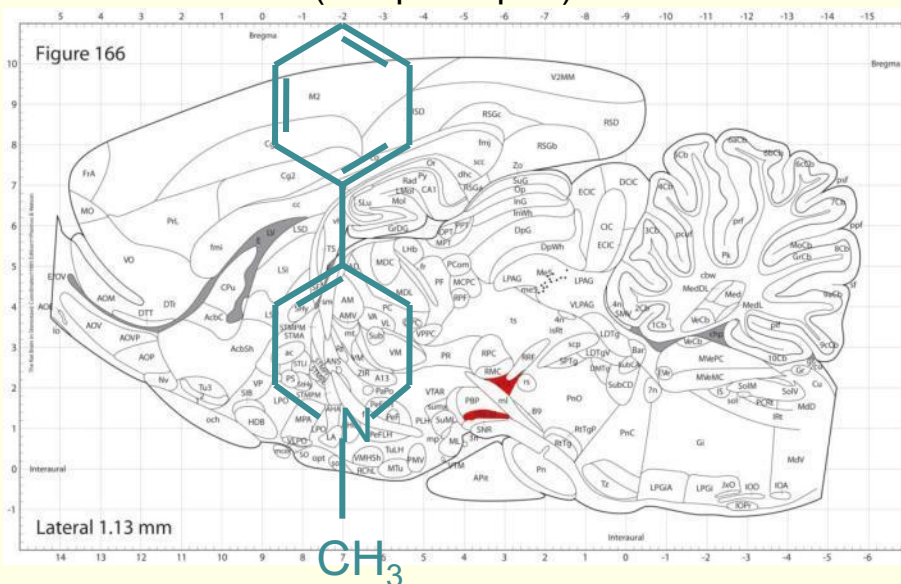
Another group of researchers looked elsewhere and confirmed that D-amphetamine destroys cells in parietal cortex and somatosensory barrel field cortex **as well as** the frontal cortex, piriform cortex, hippocampus, caudate putamen, VPL of thalamus, and (not shown): *tenia tecta*, *septum* and other *thalamic nuclei*

***Cell death can only be witnessed in locations that are assessed***

# Derivatives of the same compound can damage different locations with different effects

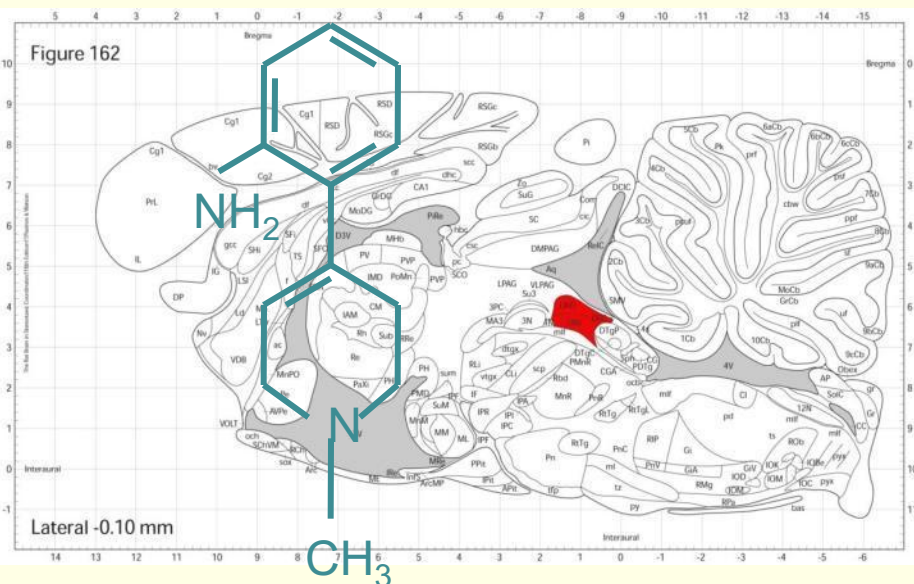
## MPTP:

destroys cells in the VTA and substantia nigra (compacta part)



## 2'-NH<sub>2</sub>-MPTP:

selectively destroys cells in dorsal raphe



MPTP damages the dopaminergic system while 2'-NH<sub>2</sub>-MPTP damages the serotonergic system

*The neurotoxic profiles of a compound cannot be predicted by known profiles of other (even similar) compounds*

# Location, location, location: summary of concepts

- ▶ The brain is heterogeneous. Each of the 600+ populations has unique functions
- ▶ Neurotoxins often affect just one or perhaps several distinct and possibly distant regions
- ▶ Affected regions can be very small, but functionally significant
- ▶ The location of effects is unpredictable:
  - ▶ Based on other pathologic and behavioral indicators
  - ▶ Between compounds that share similar structures (same class)

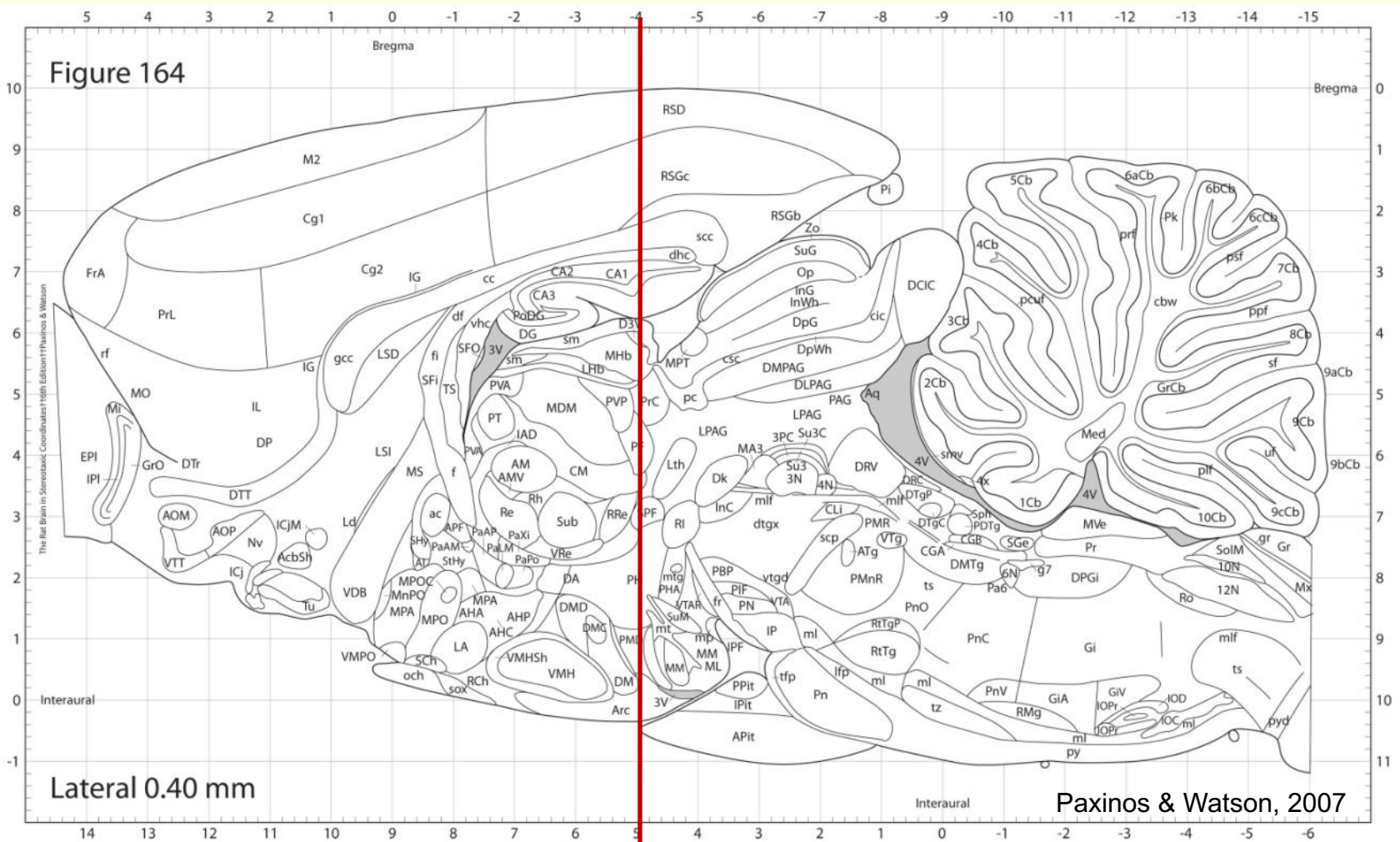
***The design of an effective safety screen addresses these spatial considerations.***

A well-defined sampling strategy addresses the spatial considerations that are necessary for routine safety assessments

- ▶ A consistent, systematic approach to sampling is the most practical
- ▶ Evaluating full cross sections of the brain (levels) at regular intervals from end to end is the recommended approach to sampling

*Defining the interval spacing between samples becomes the key to a successful designed approach*

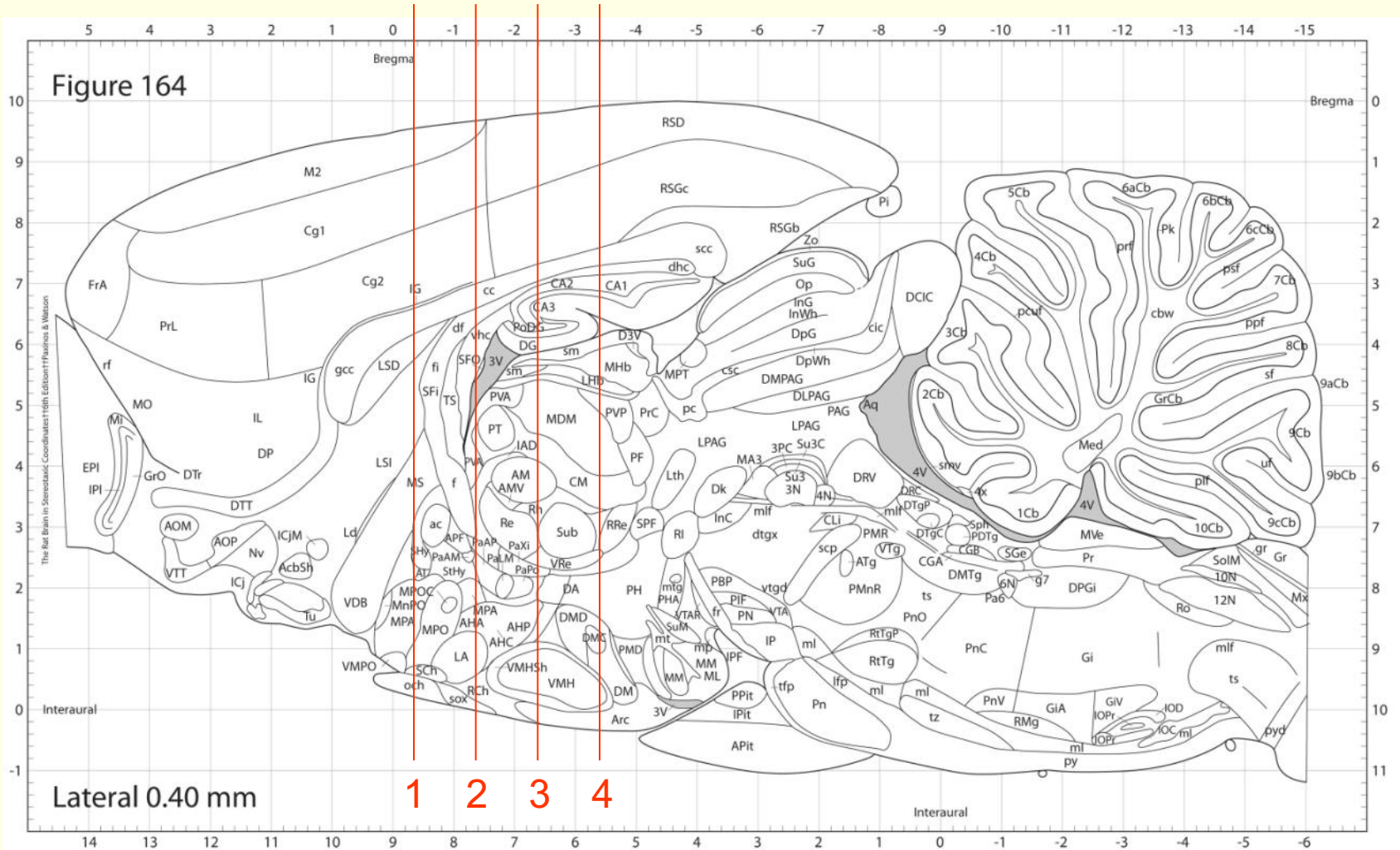
A single cross-section of the brain is called a level. Any single level crosses a relatively small % of brain cell populations



## How many levels are adequate?



The populations of the brain differ dramatically between levels  
that are separated by very short intervals

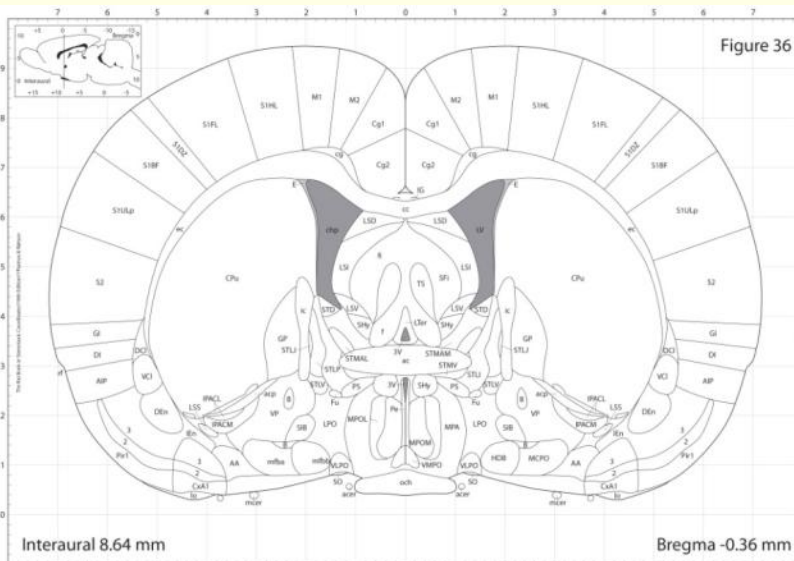


***The rat brain is ~21mm long. Let's examine the changes that occur across 1mm intervals:***

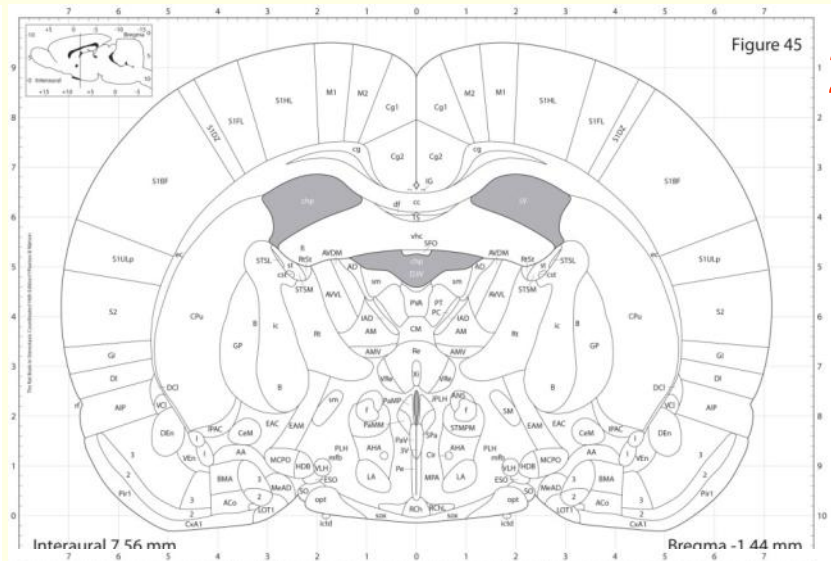


# Significant changes are easily visible just one mm between levels

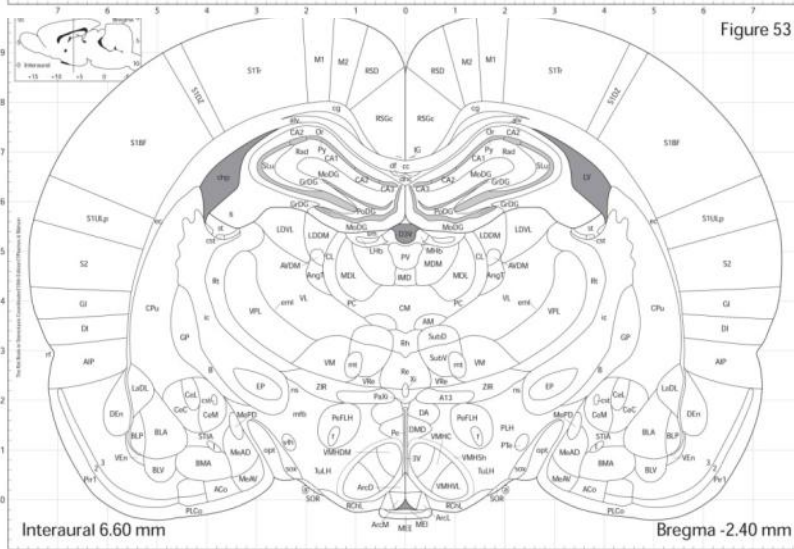
1



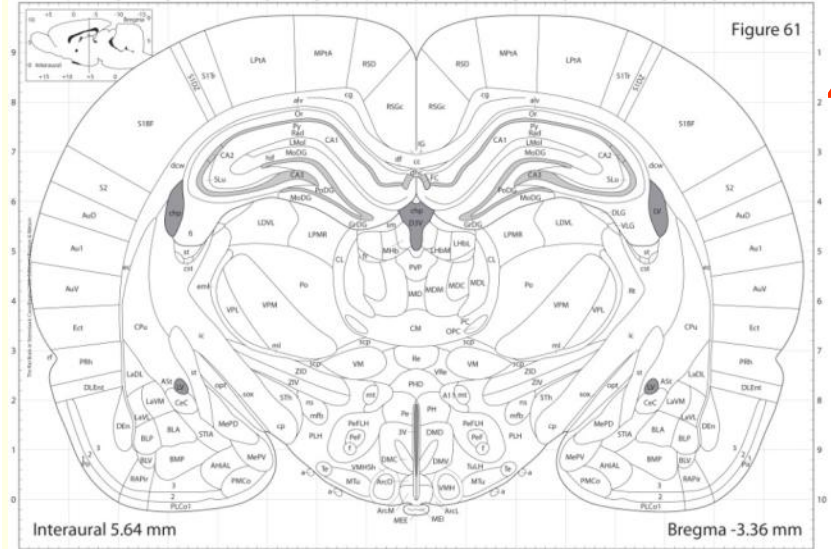
2



3



4





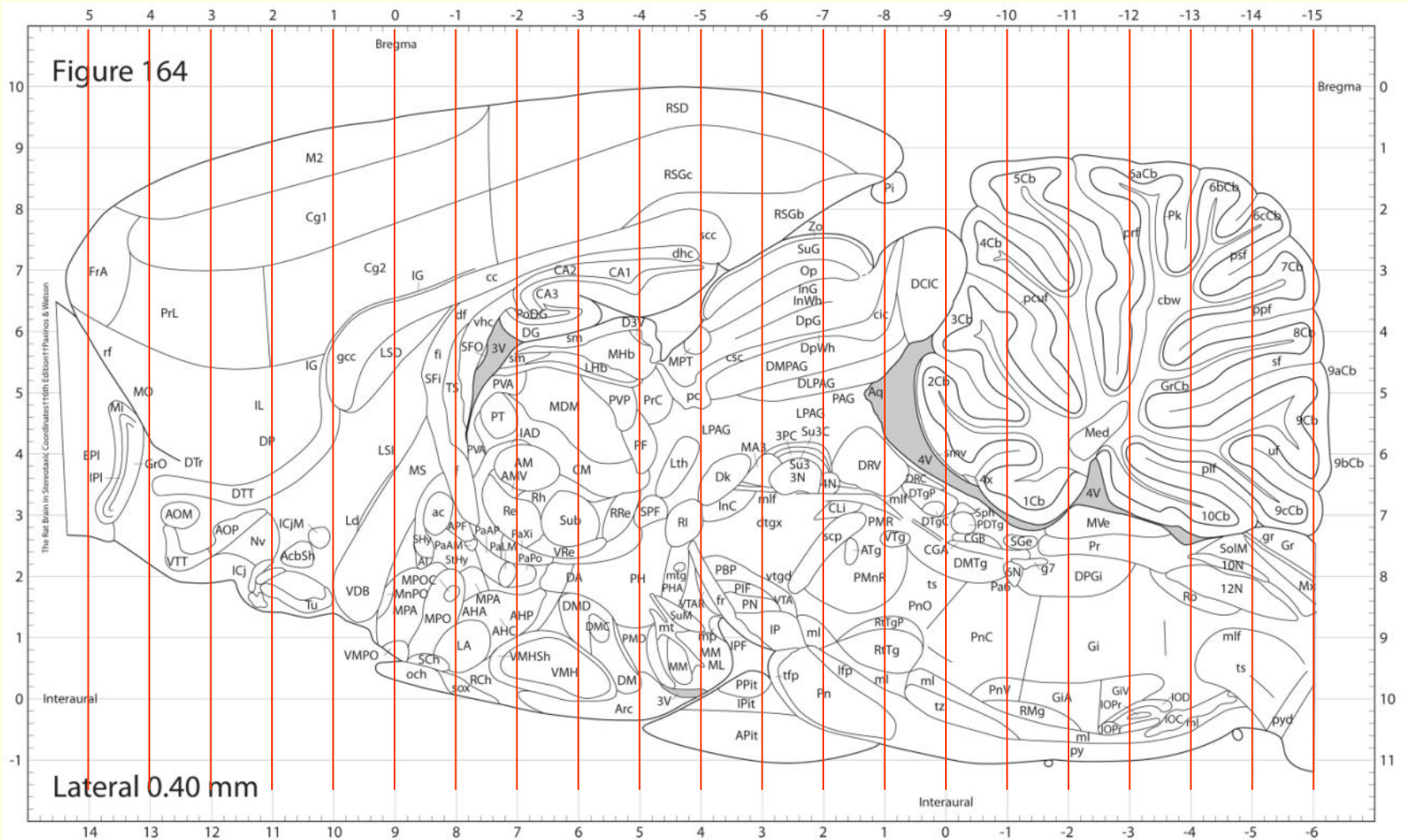
# Defining a sampling approach for routine pathologic assessments is a trade-off exercise

- ▶ To sample every adjacent level of the brain would be totally thorough, but impractical and unnecessary
- ▶ Sampling levels at too great an interval can leave gaps and populations that would not be assessed

***A compromise approach must be selected that delivers reasonable safety assurance without imposing an excessive burden on the pathologist.***

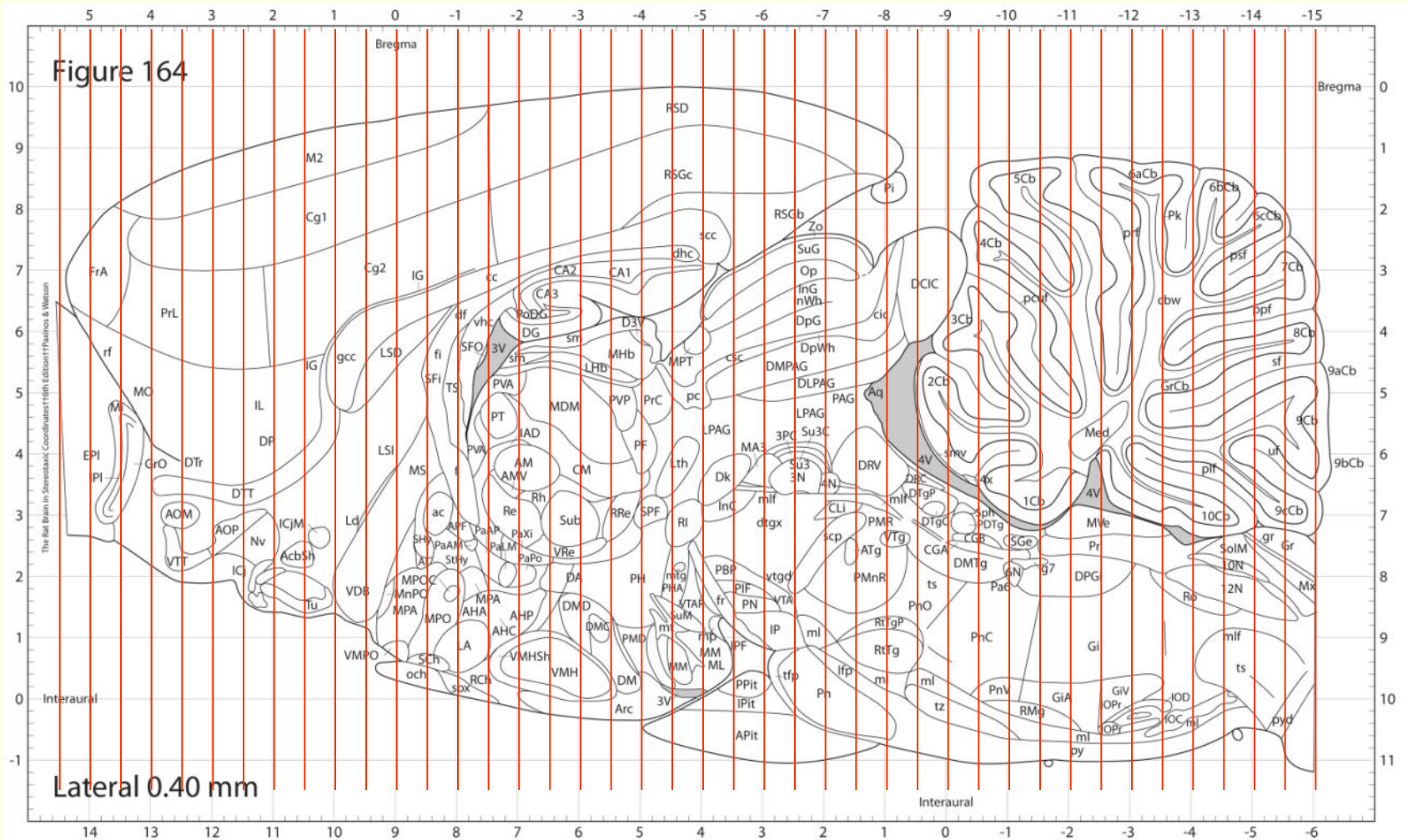


1mm intervals between levels has been shown to leave broad gaps between samples



**1mm sampling yields ~20-23 sections in a rat brain**

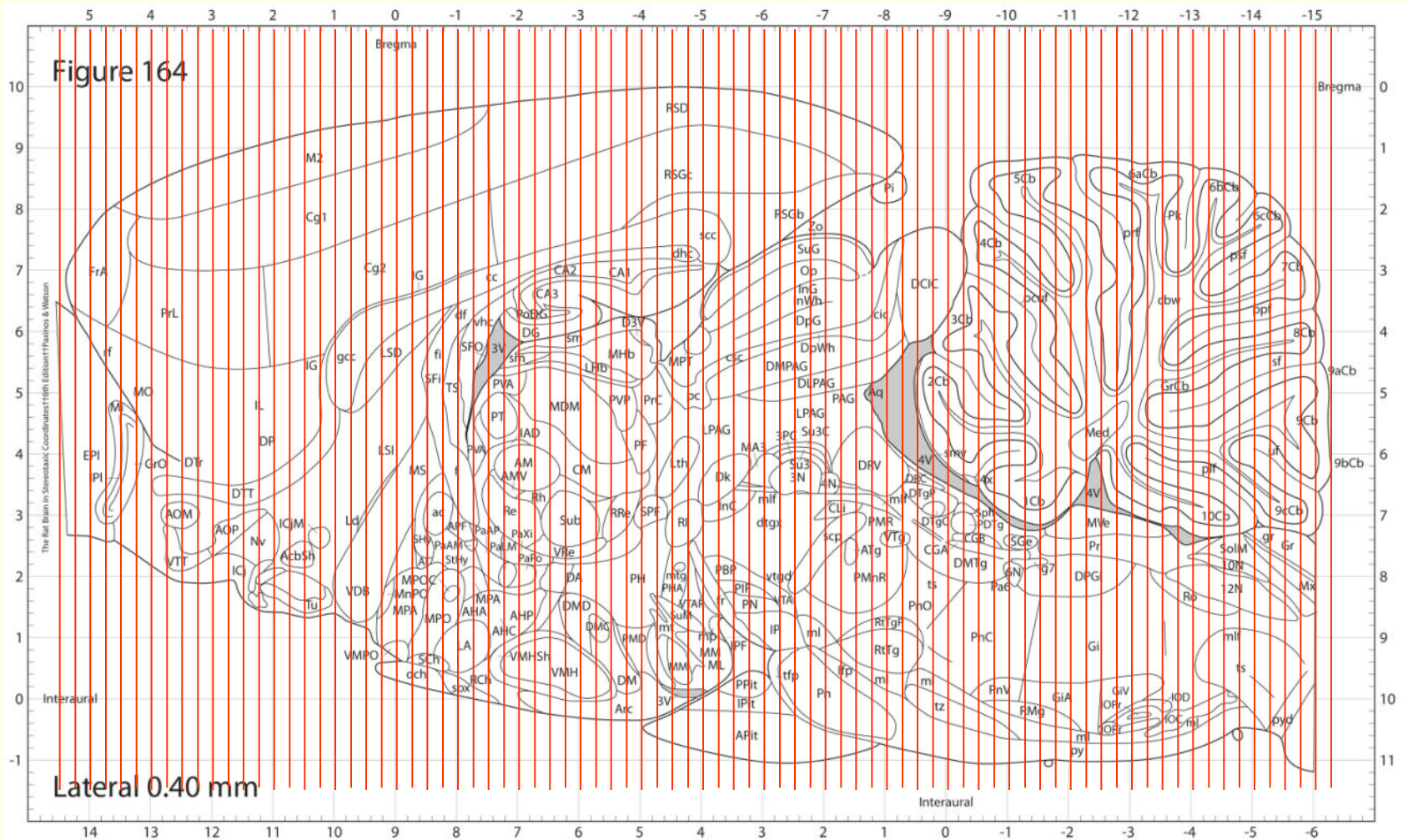
0.5mm intervals between levels greatly improve the opportunity to sample all populations, but gaps can still occur



**0.5mm sampling yields ~40-46 sections in a rat brain**

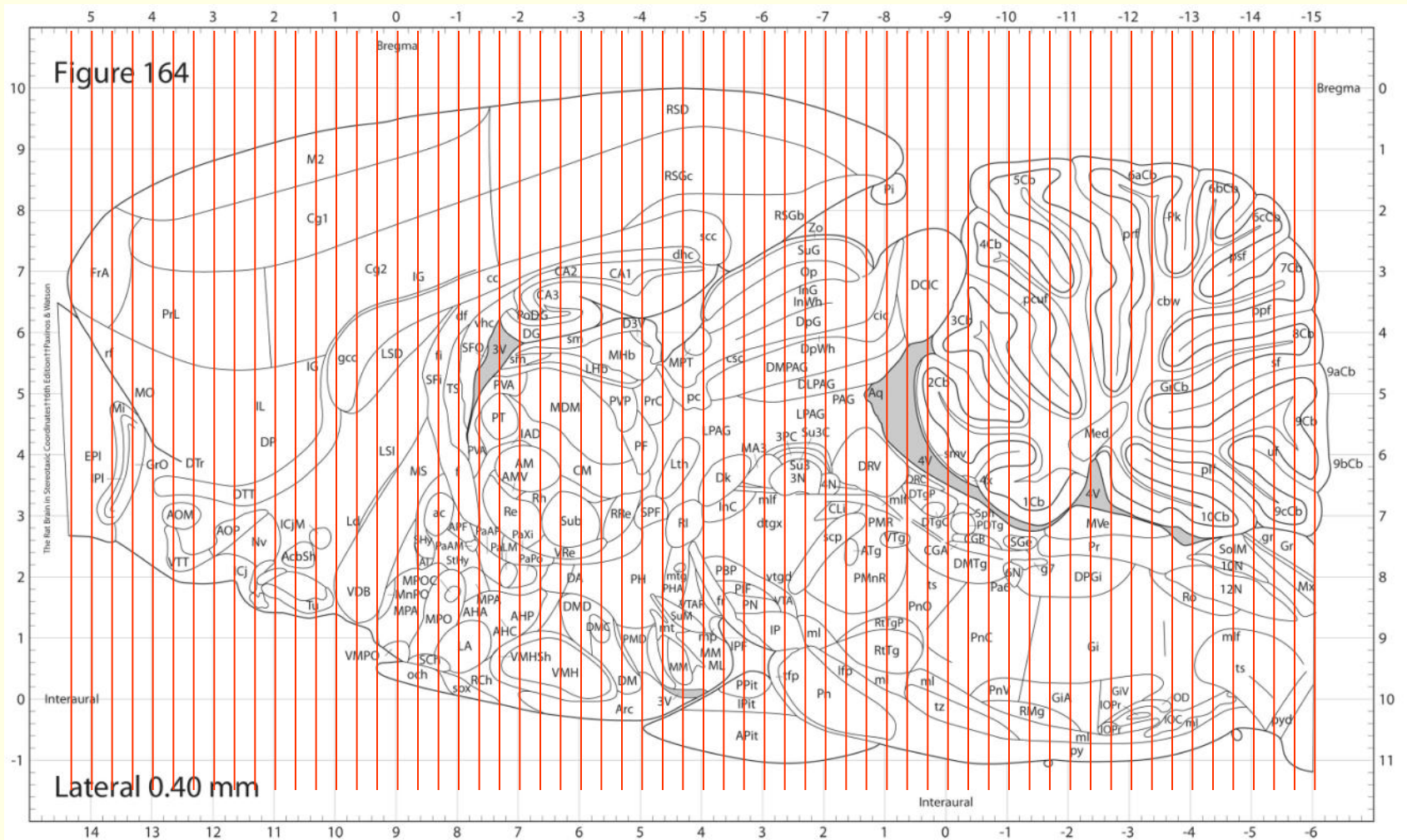


0.25mm intervals between levels is very thorough, with most populations likely to be sampled multiple times



***0.25mm sampling yields ~80-90 sections in a rat brain.  
This was the frequency reflected in the original Paxinos atlas***

0.32mm spacing between levels is the interval commonly used in R&D when characterizing effects in a rat brain



***0.32mm sampling yields ~60-65 sections in a rat brain. This spacing ensures adequate representation of most populations of the brain.***

For any species, sampling the same number of levels provides comparable representation

**Sampling “rules of thumb”**

Species	Brain Length (mm)	Sampling Interval (in mm)		
		Using 40 samples	Using 60 samples	Using 80 samples
Mouse	12	0.30	0.20	0.15
Rat	21	0.53	0.35	0.26
Monkey	65	1.63	1.08	0.81
Dog	75	1.88	1.25	0.94

*A sampling rate of 50-60 levels per brain offers a balance between a reasonable safety assessment and reasonable effort.*

When: Time-course for observations



# The time-course of cell death in the brain creates a challenge for witnessing cell death

- ▶ The “point of no return” for cell death is reached some time AFTER compound administration. The amount of time (after) can vary.
- ▶ Cell death can only be observed if observation is timed correctly following the administration of a compound
  - ▶ There is a limited period of time during which the death of any cell can be detected
- ▶ The timeline of cell death following administration of a neurotoxin varies from one compound to the next

***Despite these attributes, there are timing “rules” for cell death that make it possible to define efficient screens and/or comprehensive safety tests***

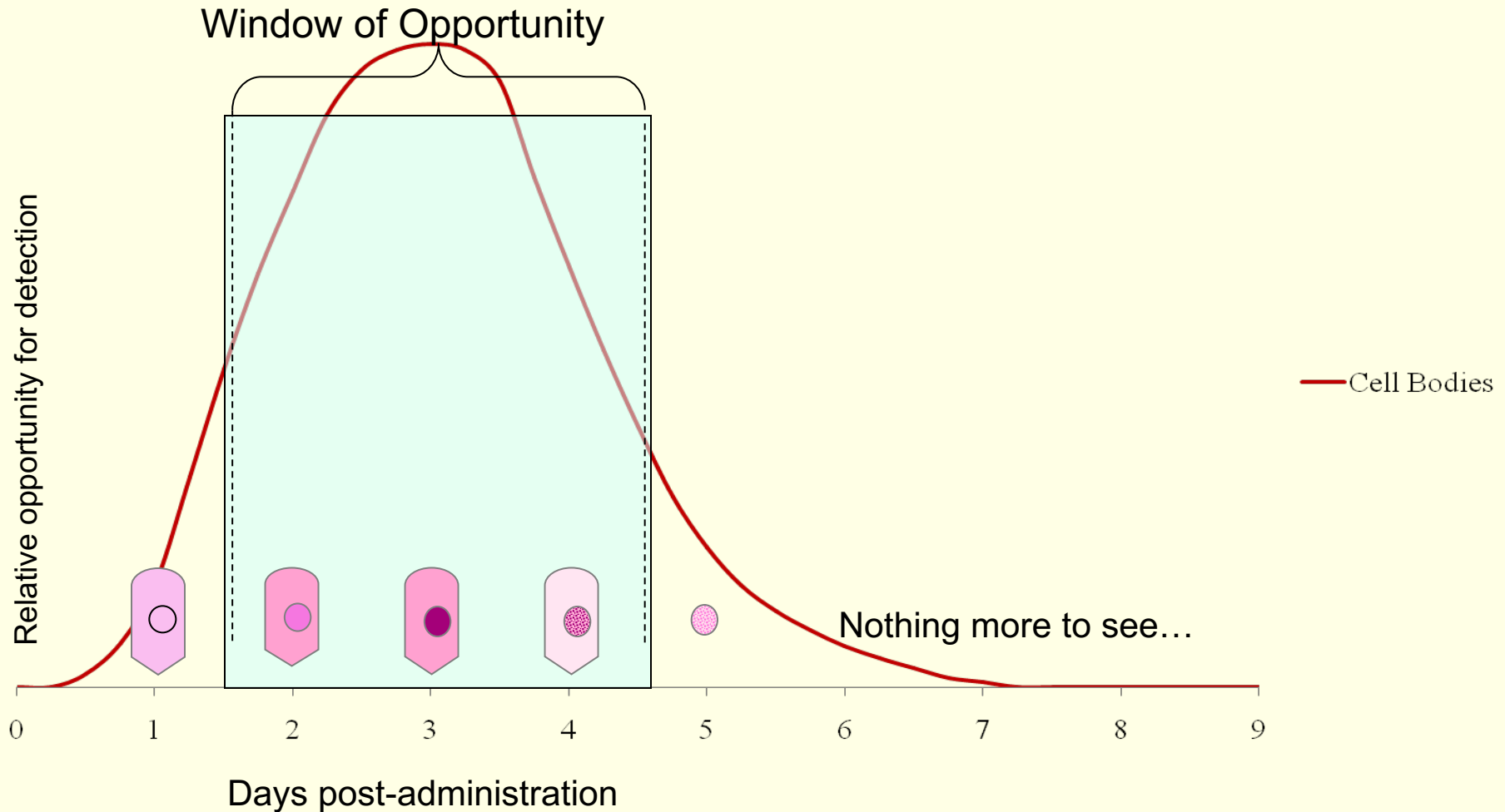


# Cell death due to acute exposure has predictable characteristics and timing

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

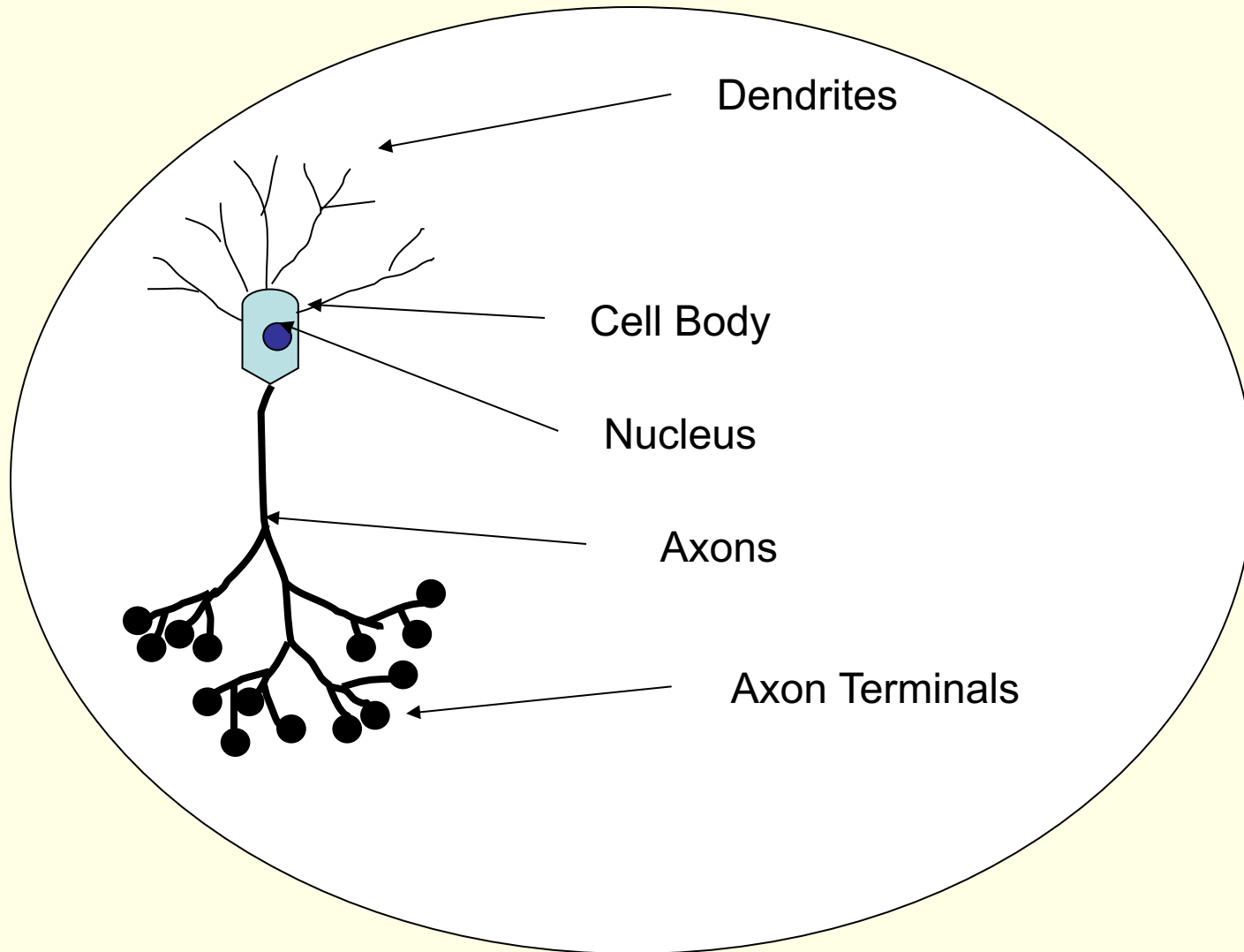
***The consistent tendencies of acute cell death enable reliable screening approaches to be used***

# Cell death from an acute response to a compound follows a reliably consistent time-course

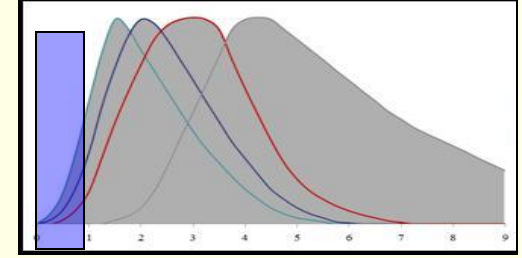


***The window of opportunity for viewing a cell death event lasts ~3 days***

# Time Lapse Model of Neurodegeneration

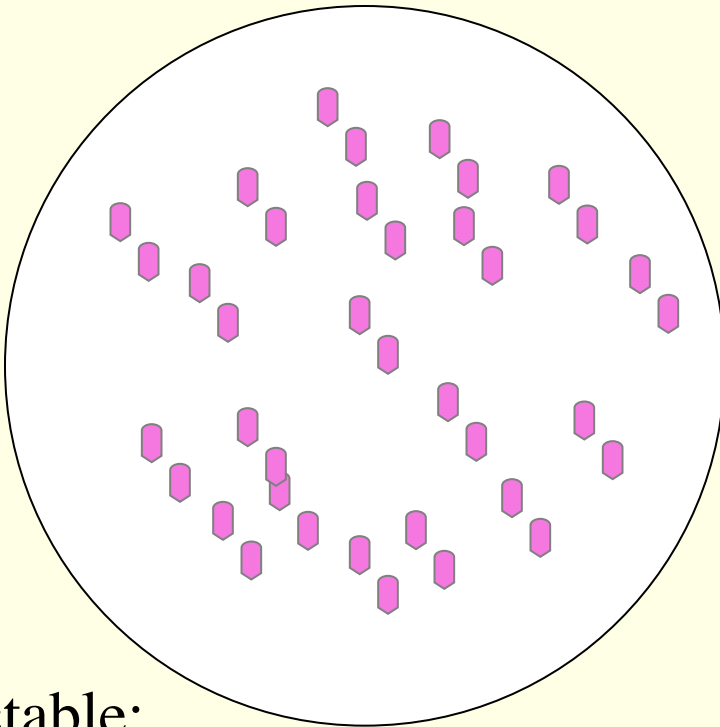


# Day 0



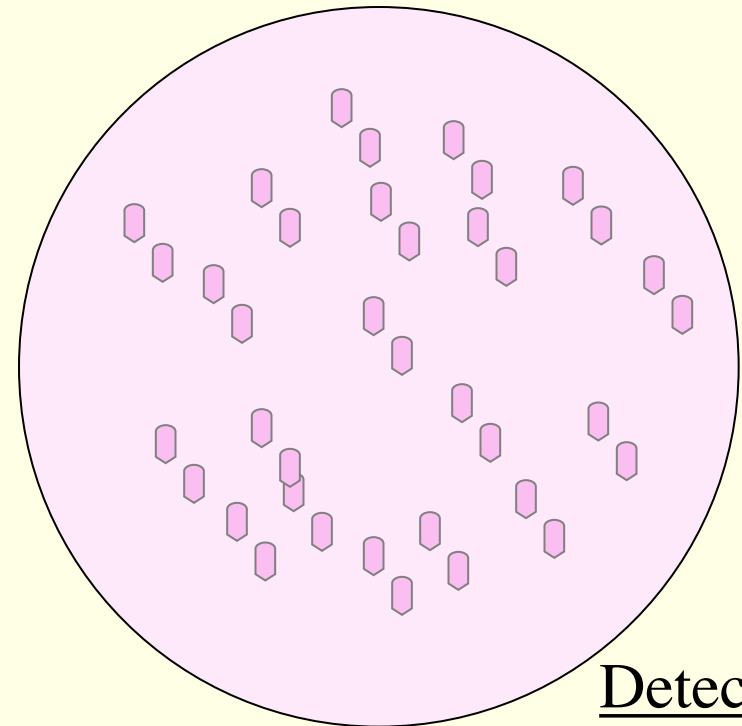
Only normal cells Detectable

Only normal cells Detectable



Detectable:

Healthy cells



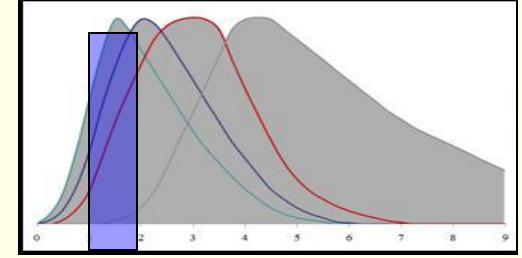
Detectable:

Healthy Cells

Footprint of all elements

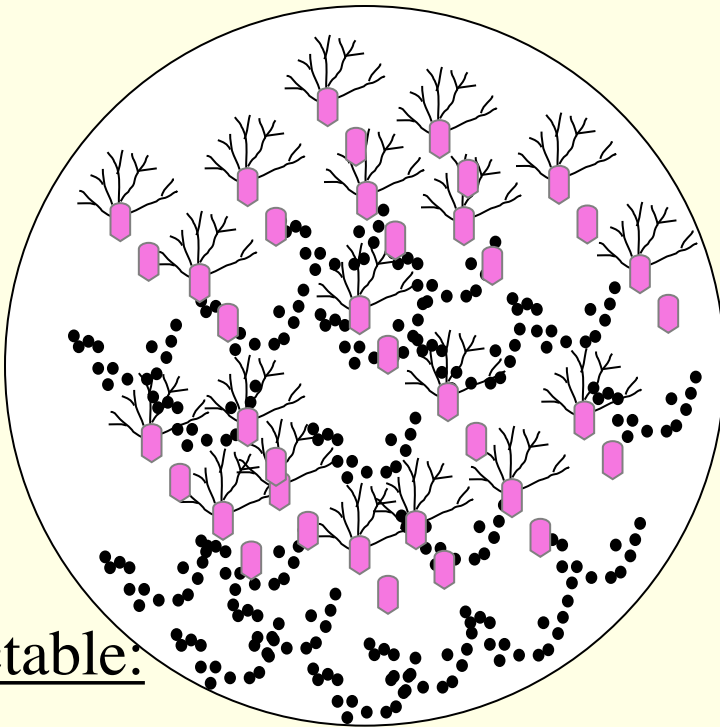
Footprint of cell body (only)

# Day 1



Disintegrating dendrites and synaptic terminals appear

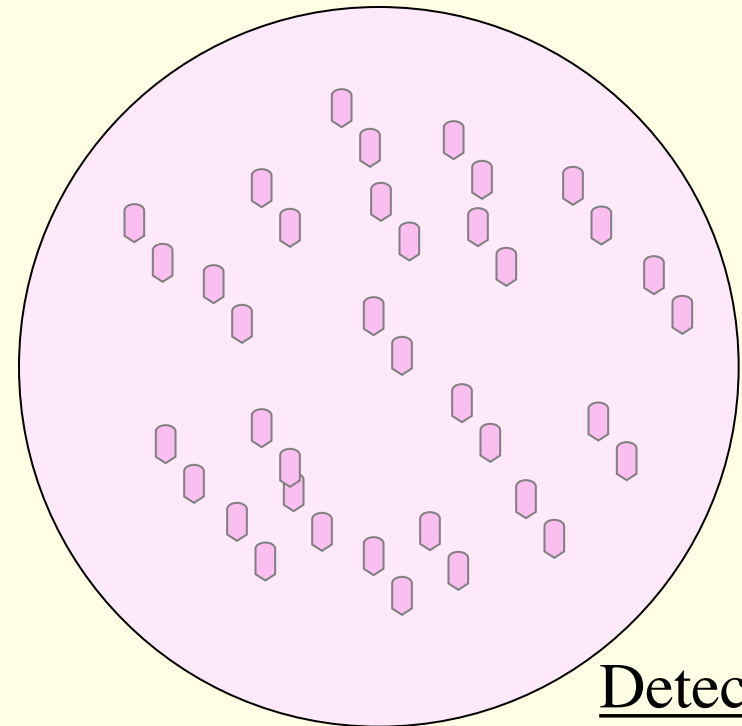
All cells appear normal



Detectable:

Dendrites  
Synaptic Terminals

Footprint of all elements



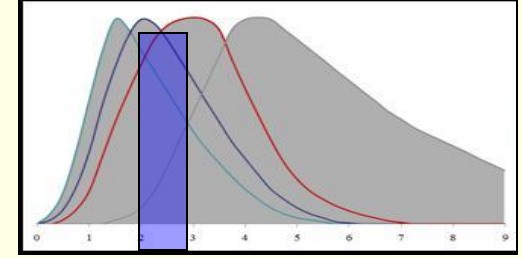
Detectable:

Healthy Cells

Footprint of cell body (only)

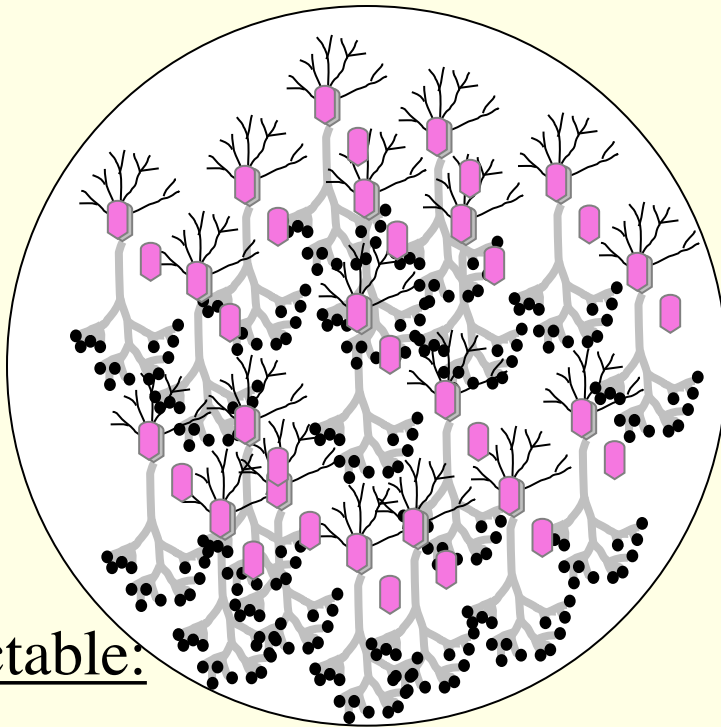


# Day 2



Disintegrating cell bodies and axons appear

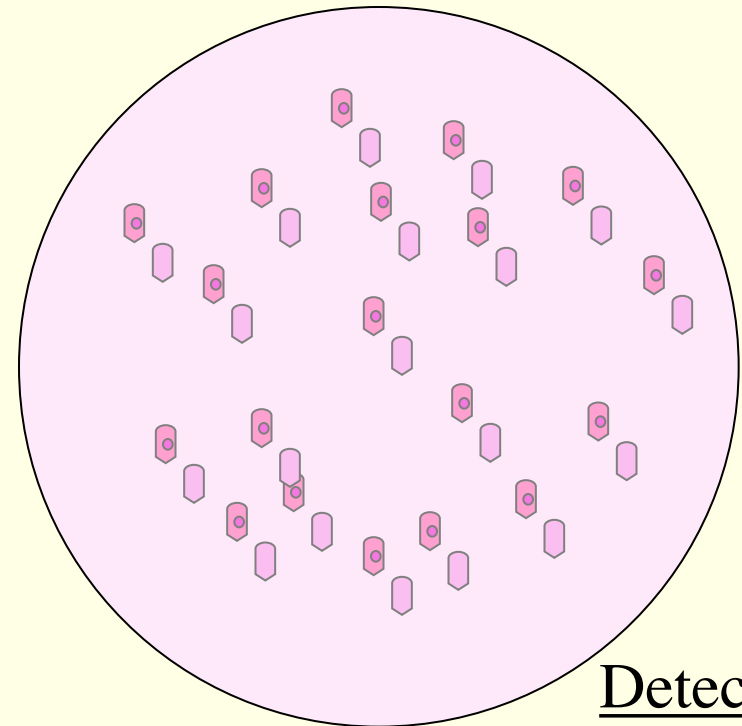
Nucleus of disintegrating cell bodies becomes Detectable



Detectable:

Dendrites  
Synaptic Terminals  
Cell Bodies  
Axons

Footprint of all elements

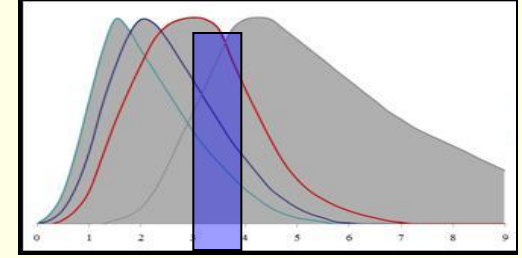


Detectable:

Cell Bodies

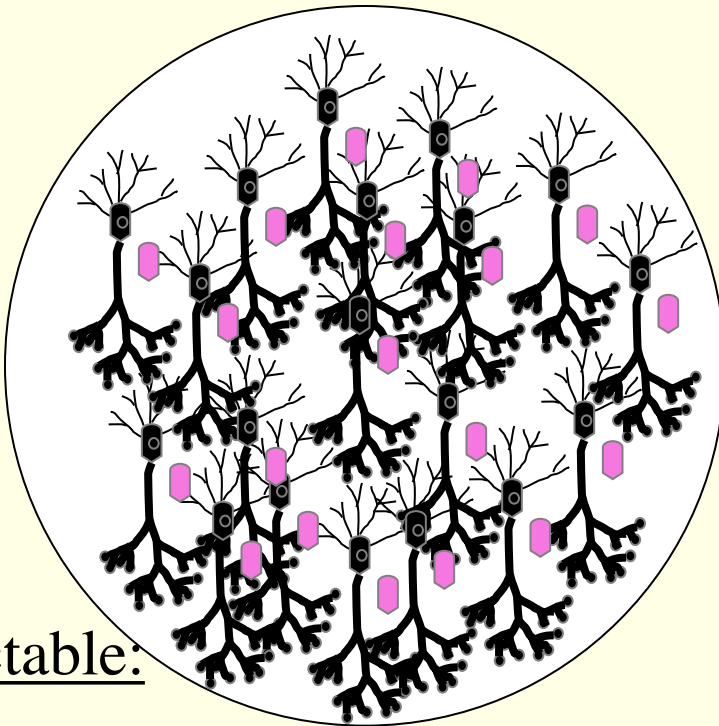
Footprint of cell body (only)

# Day 3



All elements are Detectable

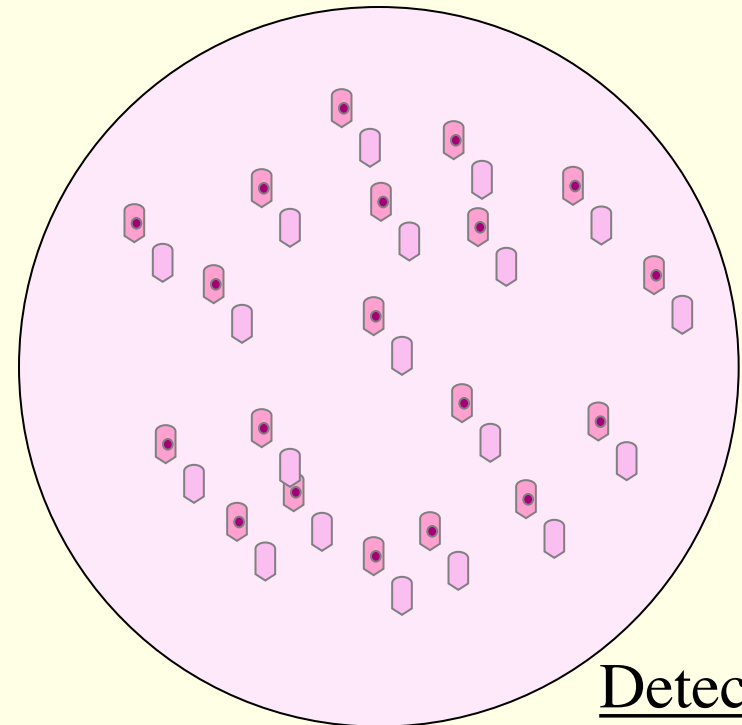
Nucleus of disintegrating cell bodies remains Detectable



Detectable:

Dendrites  
Synaptic Terminals  
Cell Bodies  
Axons

Footprint of all elements

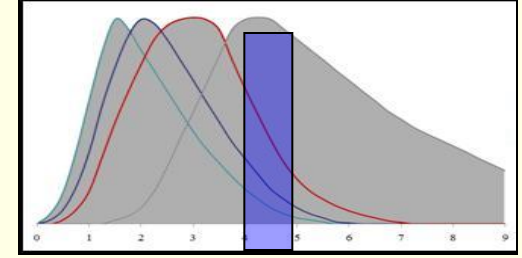


Detectable:

Cell Bodies

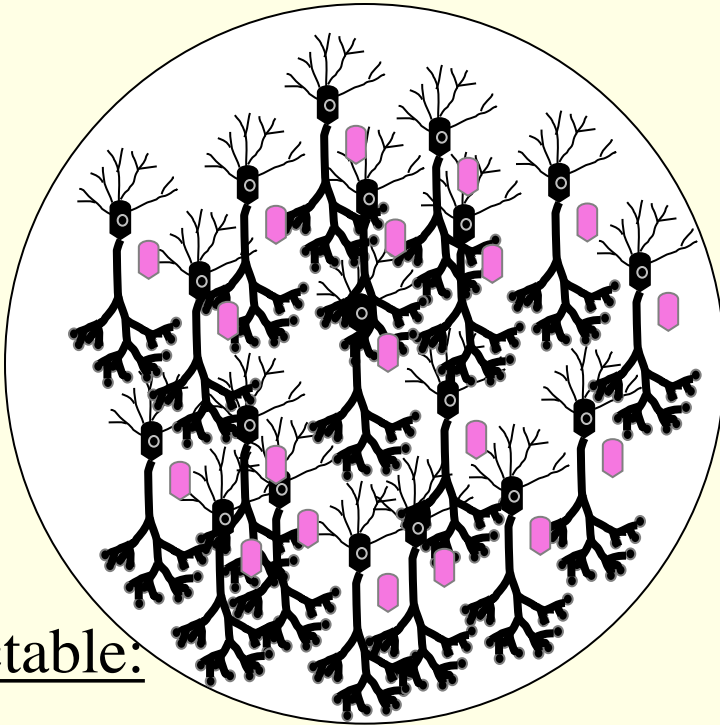
Footprint of cell body (only)

# Day 4



Synaptic terminal signal  
dissipates

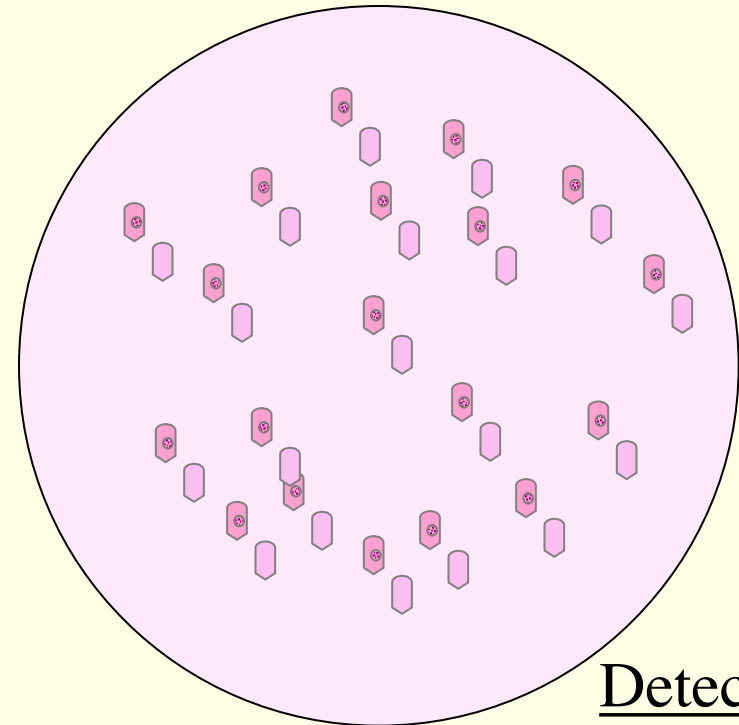
Nucleus begins to fragment



Detectable:

Dendrites  
Cell Bodies  
Axons

Footprint of all elements

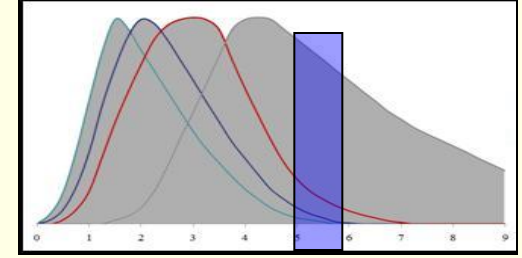


Detectable:

Cell Bodies

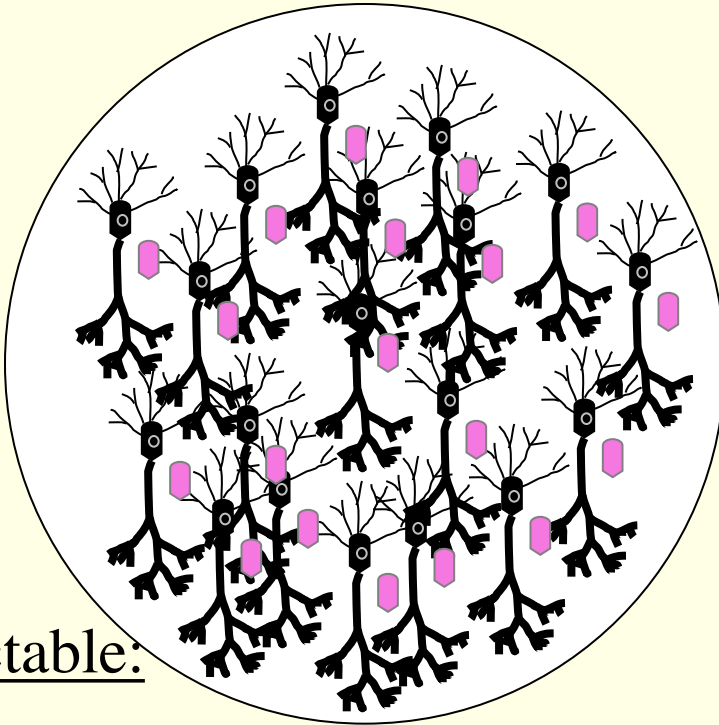
Footprint of cell body (only)

# Day 5



Dendrite debris is removed  
Cell Body debris is removed

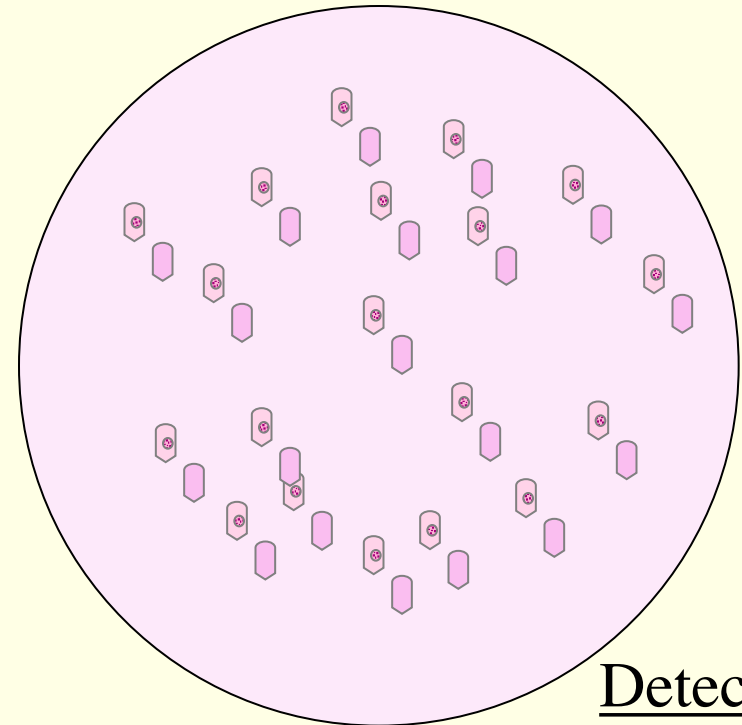
Cell Body debris is removed



Detectable:

Dendrites  
Axons

Footprint of all elements



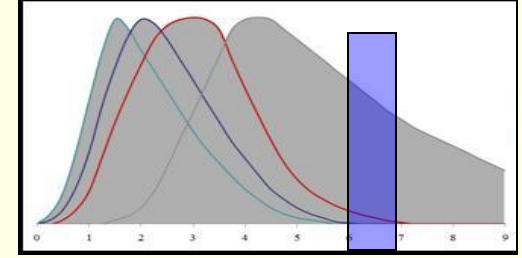
Detectable:

Healthy Cells

Footprint of cell body (only)

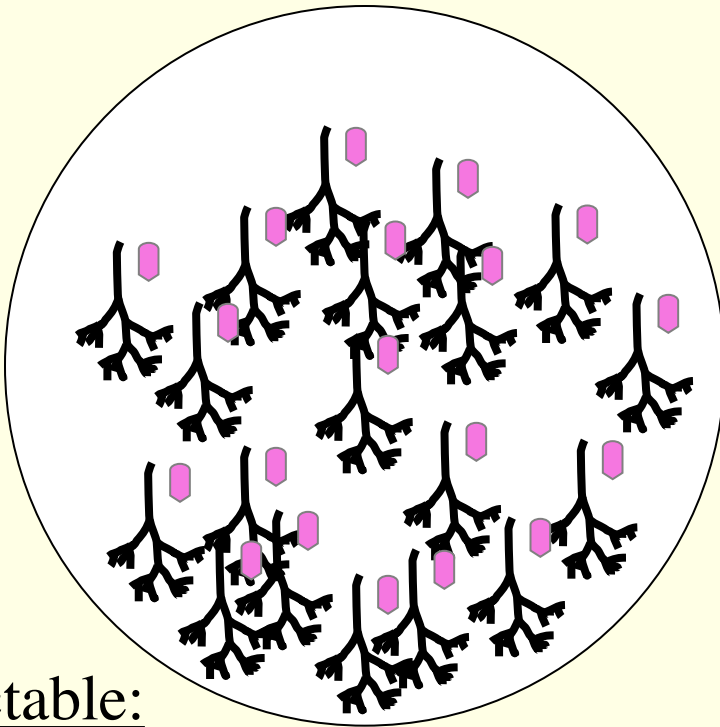


# Day 6



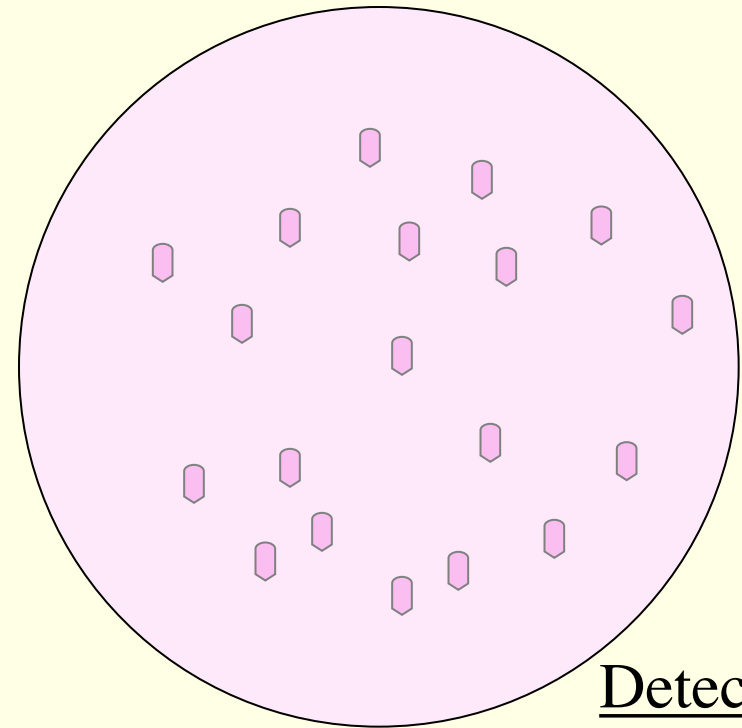
Disintegrating Axons remain

No debris to detect



Detectable:  
Axons

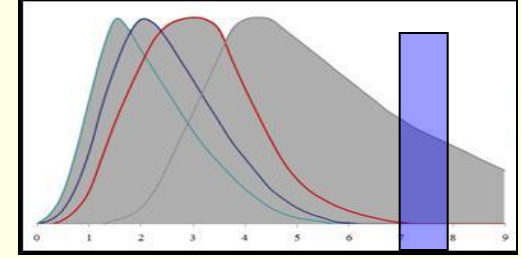
Footprint of all elements



Detectable:  
Healthy Cells

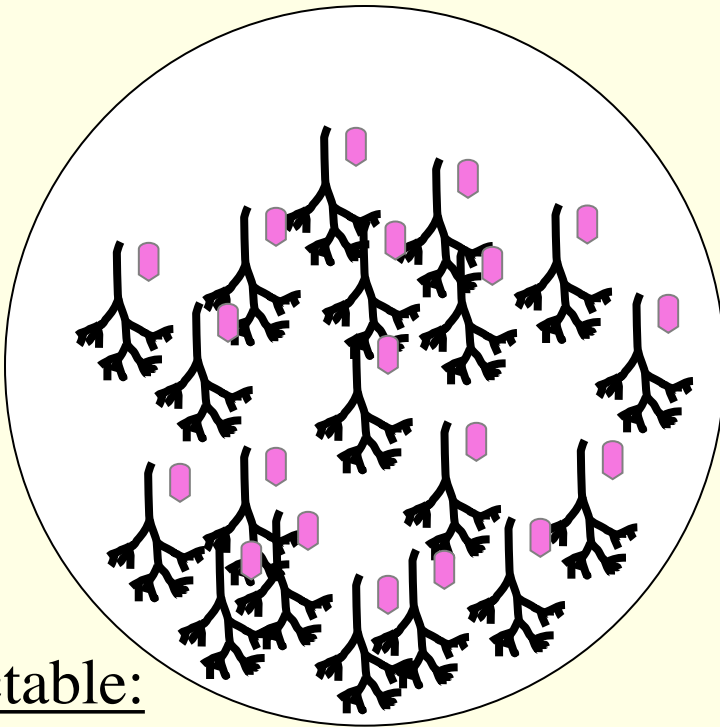
Footprint of cell body (only)

# Day 7



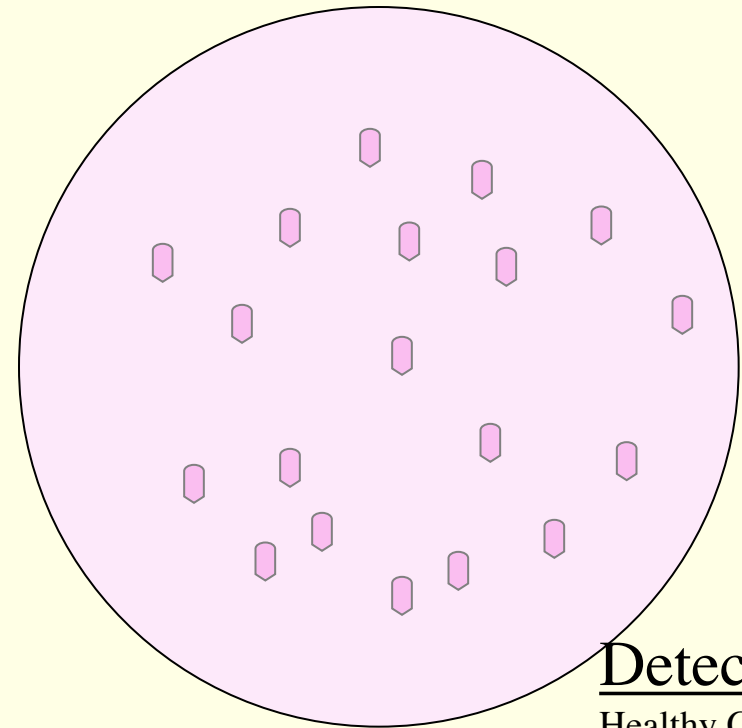
Disintegrating Axons remain

No debris to detect



Detectable:  
Axons

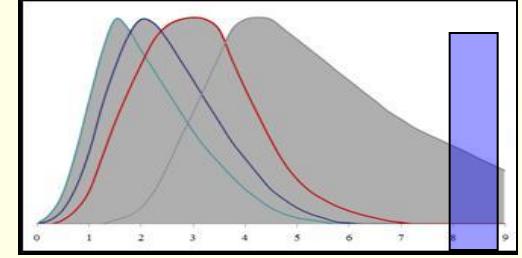
Footprint of all elements



Detectable:  
Healthy Cells

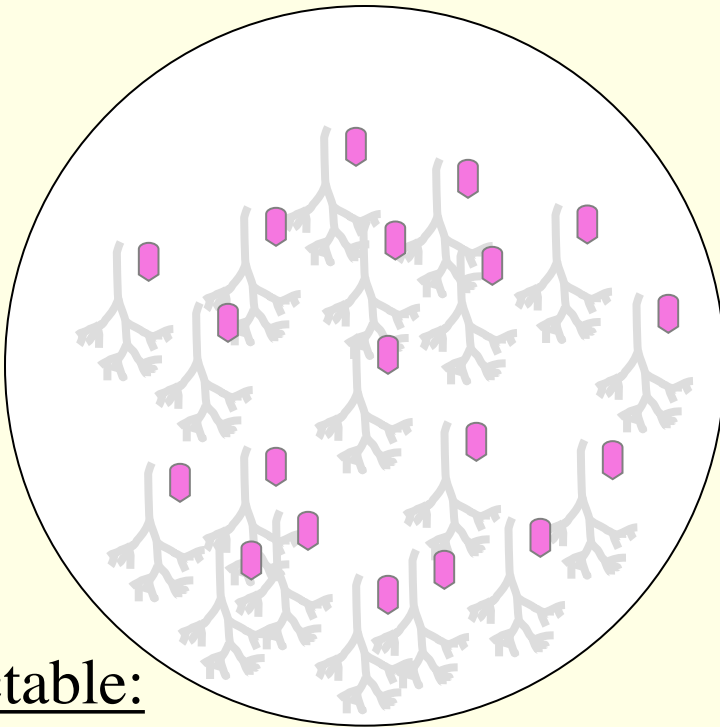
Footprint of cell body (only)

# Day 8+



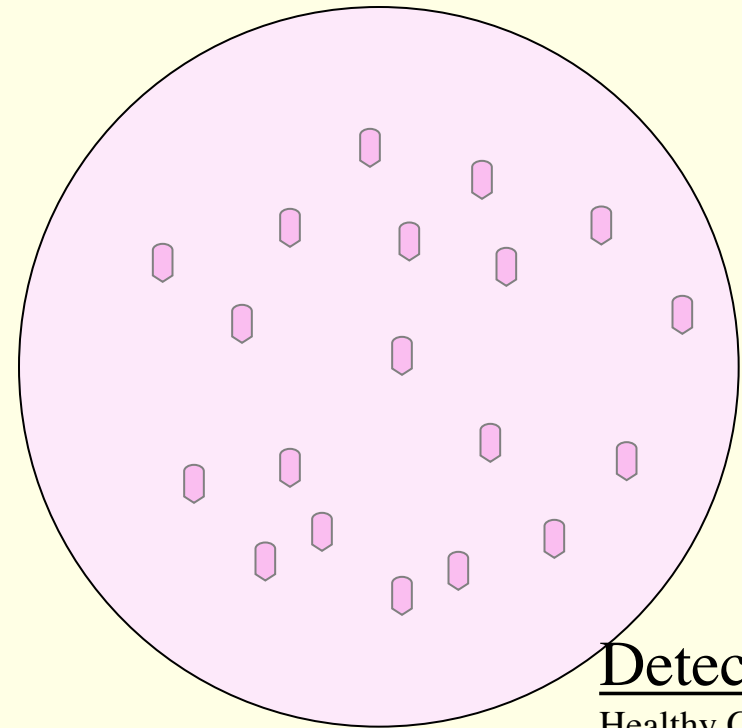
Axon debris is removed beyond  
8 days

No debris to detect



Detectable:  
Axons

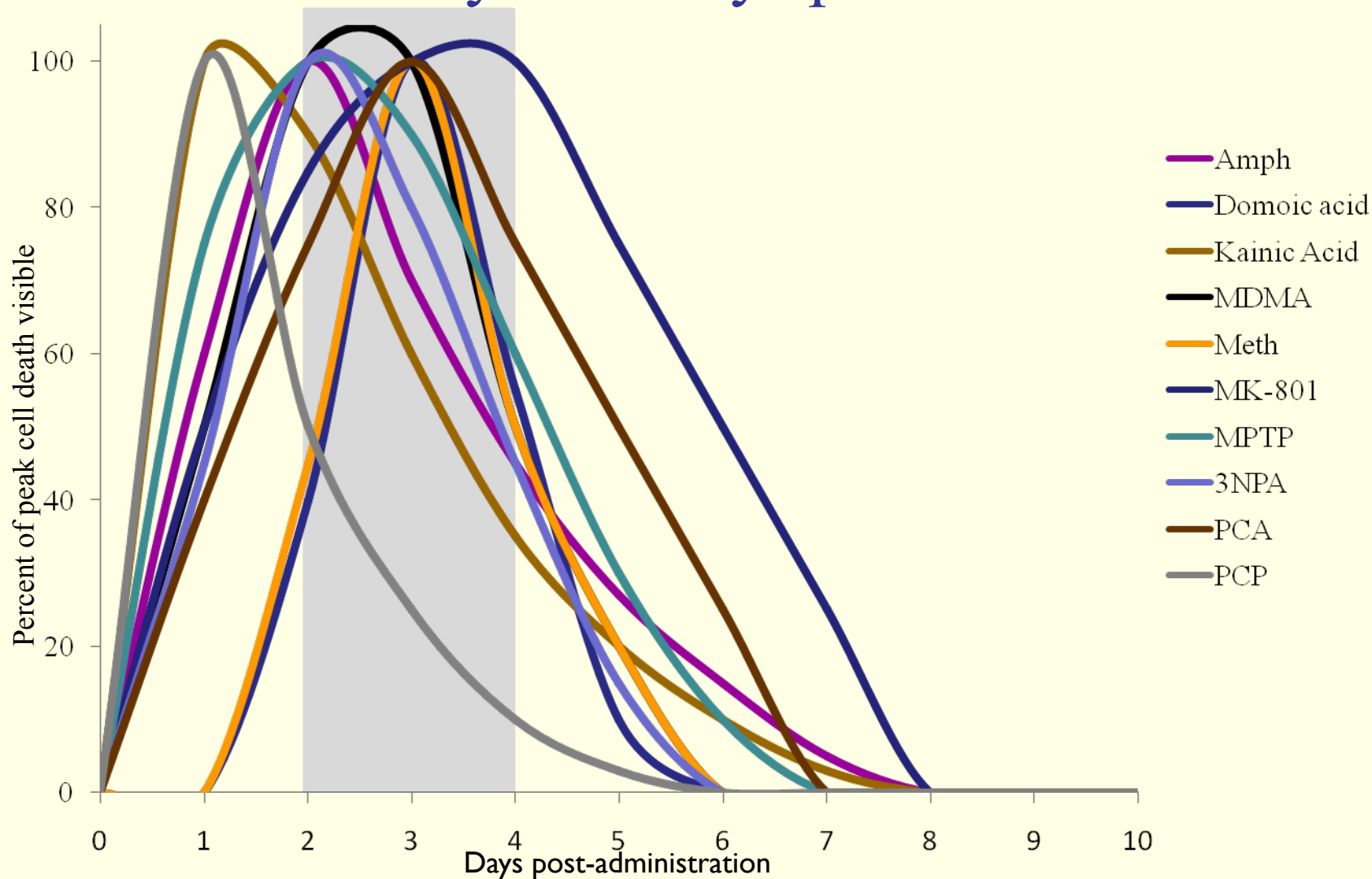
Footprint of all elements



Detectable:  
Healthy Cells

Footprint of cell body (only)

The window of opportunity to observe peak cell death is usually ~2-4 days post-administration



***Evidence of cell death is transient***



# The evidence of cell death is a transient event

- ▶ Pathologic examinations reveal a snapshot in time, not a cumulative picture of past events
- ▶ Unlike cells in other organs, there is no scarring or cell replacement as past event indicators
- ▶ After the window of opportunity closes, destroyed neurons are no longer visible
- ▶ Once neurons are destroyed, they are not replaced

***While the observable evidence is transient, the effects of cell death are permanent***

Within the “probability of being observed” range  
specific timing of cell death can vary

A variety of factors can skew the observability  
curve earlier or later in the timeline:

- ▶ Each compound can illicit different pathways leading to cell death and therefore has a unique timing profile
- ▶ Higher doses can sometimes accelerate the pathway events leading to cell death
- ▶ Species, strain, gender and age can all impact the observability curve

***There is not a single time point at which all compounds will have an observable effect resulting from acute neurotoxins***

False-negative results for neurotoxicity can easily be concluded if the unpredictability of timing is not understood

► Case Study:

► Shauwecker and Steward (1997) *PNAS* :

- In a comparison of several inbred mouse strains, researchers published that C57BL/6 and BALB/c strains were “resistant” to kainic acid-induced neurotoxicity

► Benkovic, O’Callaghan and Miller (2004) *Brain Research*:

- In a later study, researchers demonstrated that those strains were NOT resistant to kainic acid-induced neurotoxicity

***Why were the results different?***

# Case Study Results: Different time points provided different data

- ▶ Dosing levels and compound administration were consistent, so why were the results different?
  - ▶ The 1997 study assessed for cell death at 4,7,12 and 20 days
  - ▶ The 2004 study assessed for cell death at 12hr, 24hr, 3 and 7 days.
  - ▶ In the 2004 study, evidence of cell death was observed to be “dramatically attenuated by 3 days following administration”... presumably removed by 4 days, leaving only normal, healthy cells
  - ▶ Both studies confirmed a lack of observable evidence by 7 days
- ▶ The lack of observable cell death at a specific point in time is not definitive. Rather, such a finding should be qualified as “not evident *at that point in time.*”

***An accurate conclusion that no cell death occurred is appropriate when all applicable times have been assessed***

# Temporal observation strategies for neuropathology are similar to other strategies for other assessments

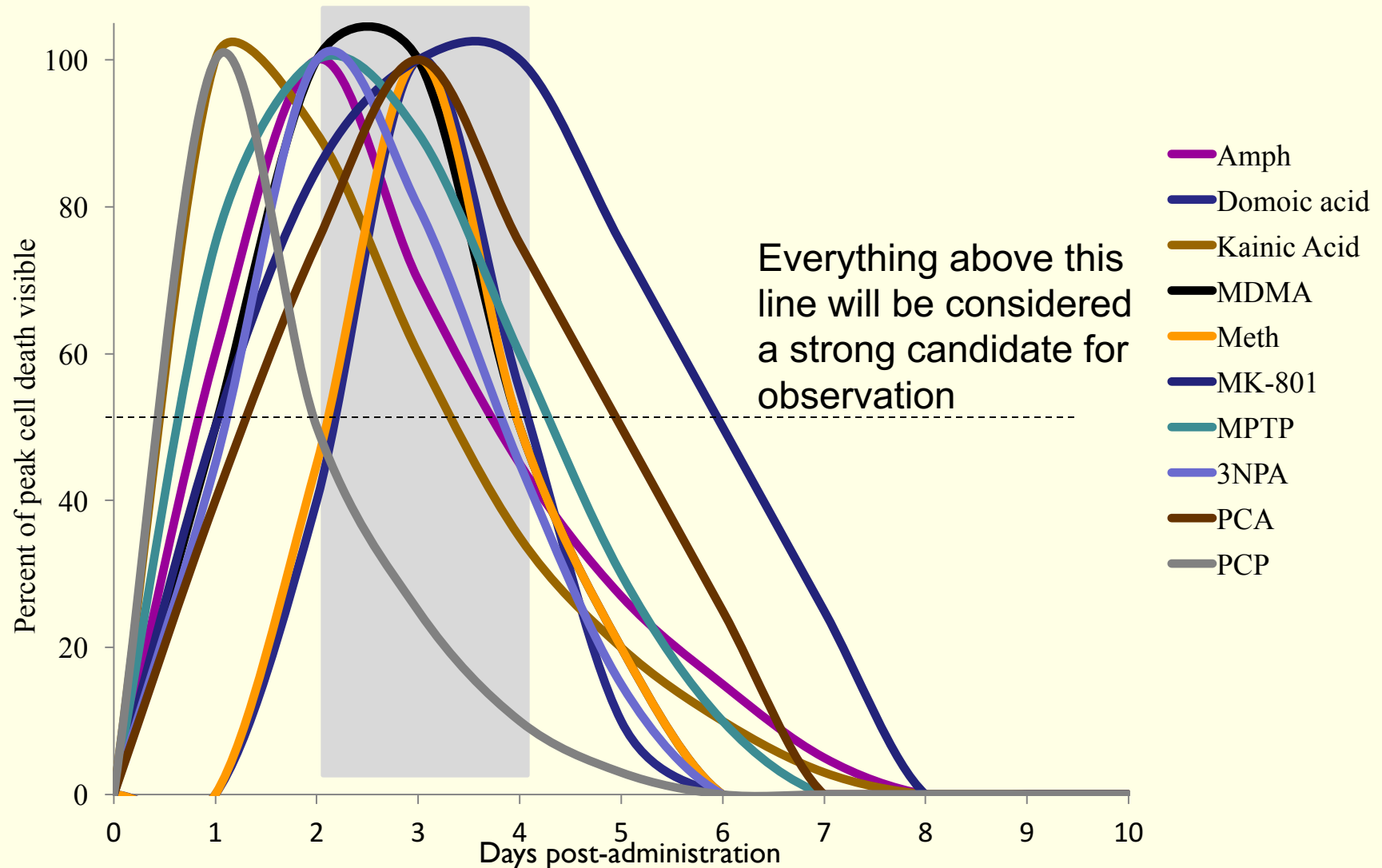
Observation for a time period is interpreted as observing DURING that time period (not just at the end):

- ▶ PK analysis require sampling over time to tell a complete story
- ▶ Functional tests, cage-side observations, FOB's, etc. are conducted throughout a study duration
- ▶ Neuropathologic “observation” entails sacrificing and assessing the brains of animals at periodic intervals during the course of a study

***Different observations require unique timing intervals for appropriate assessments/ measurements. Neuropathology has it's own appropriate temporal sampling strategy.***



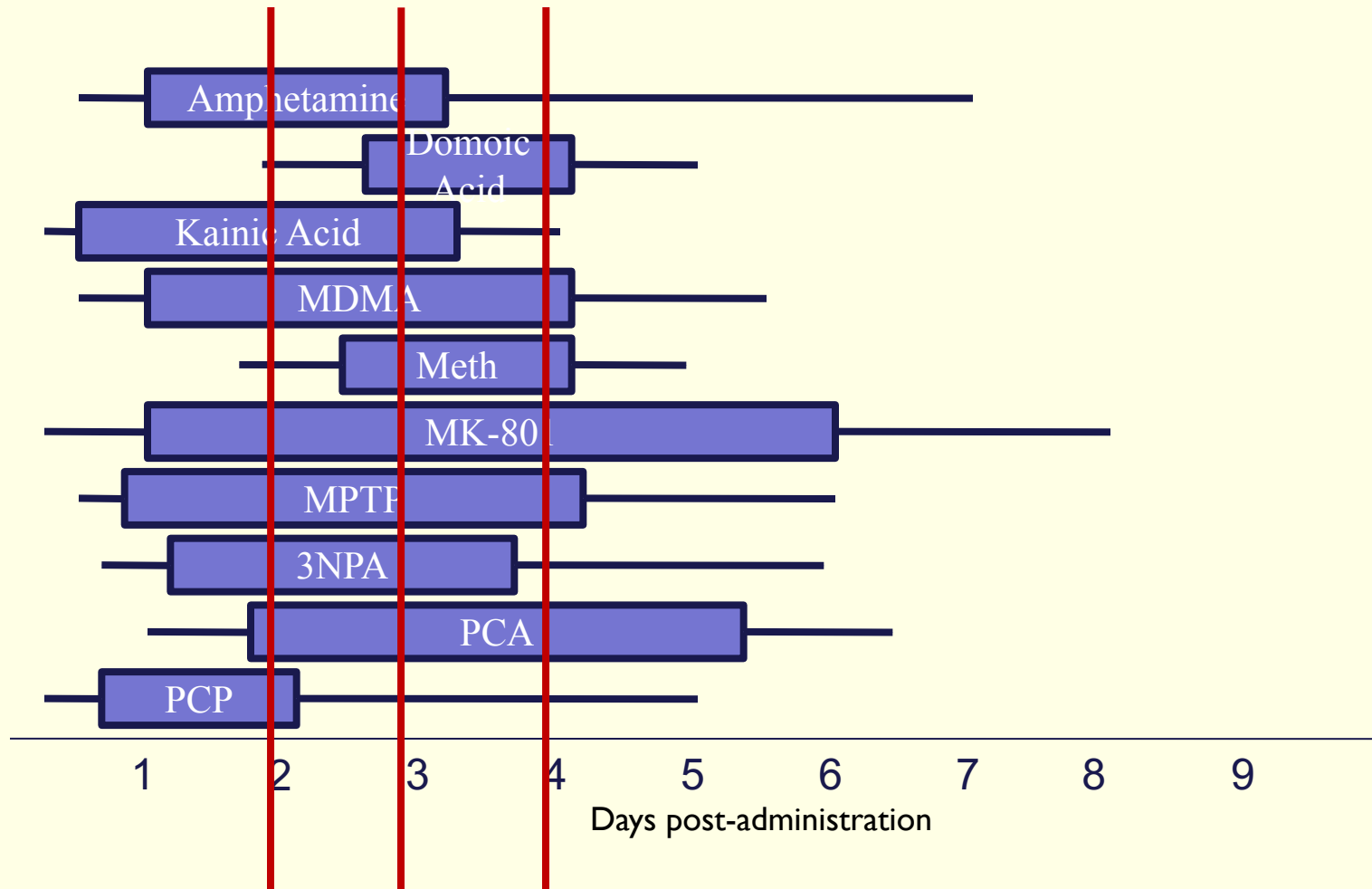
# Each compound has its own peak opportunity for detectability



Everything above this line will be considered a strong candidate for observation

***Evidence of cell death is transient***

For most of the compounds discussed in the example, there is overlap between peak opportunity for detection



***Two sacrifice times are necessary to capture both the early and late cell death cycles. Assessing a group of animals at ~48 hours and ~96 hours creates the highest probability of witnessing acute cell death.***

**COMMON STAINS:  
WHICH ARE CANDIDATES  
FOR BASELINE TESTING?**

# Detection methods for neurodegeneration

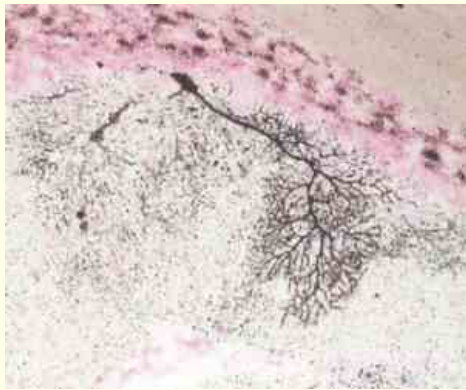
A limited number of stains are capable of detecting cell-death directly. Of these the category of degeneration specific stains are the most efficient

Recommendations are based on efficiency and accuracy. An example comparing H&E (general safety stain) and Amino CuAg (degeneration specialty stain) follow:

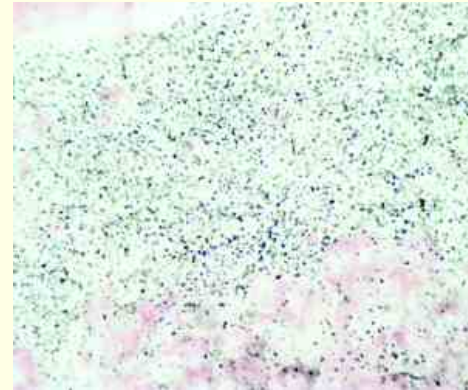


# Disintegrative Degeneration Stain

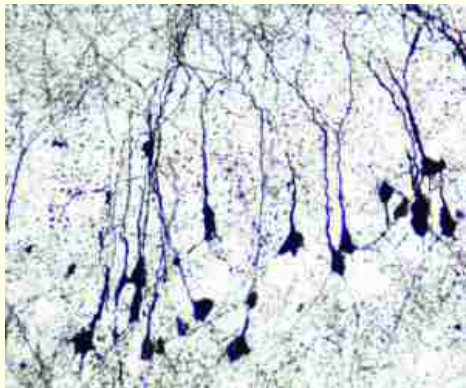
- ▶ deOlmos Amino CuAg method
- ▶ Degenerating elements stain black against white background
- ▶ Stains all degenerating neuronal elements



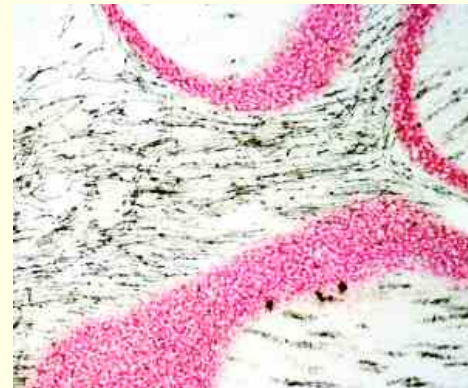
Degenerating dendrites



Degenerating synaptic terminals



Degenerating neurons



Degenerating axons

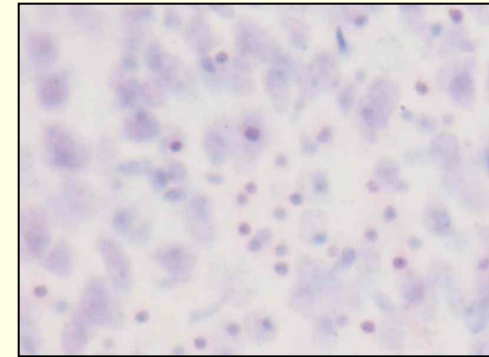
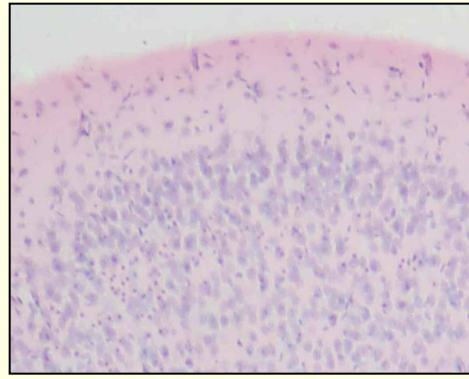
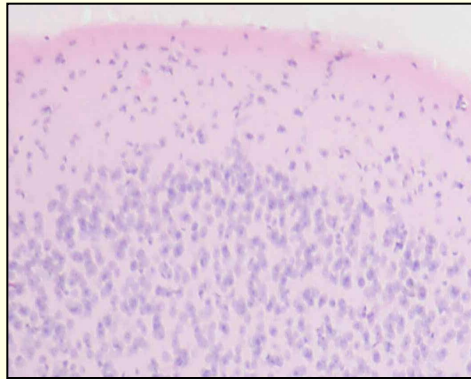
# Disintegration Stain Yields Superior Signal to Noise vs. Cellular stain

Control Case

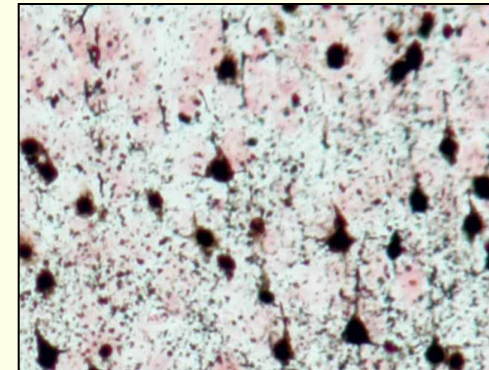
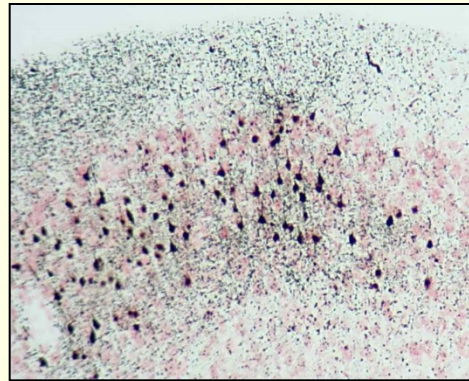
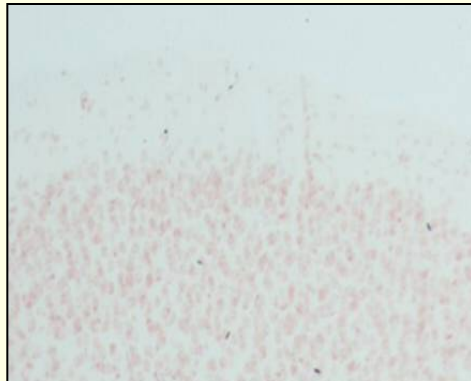
Affected Case

Higher Mag.

H&E  
Stain



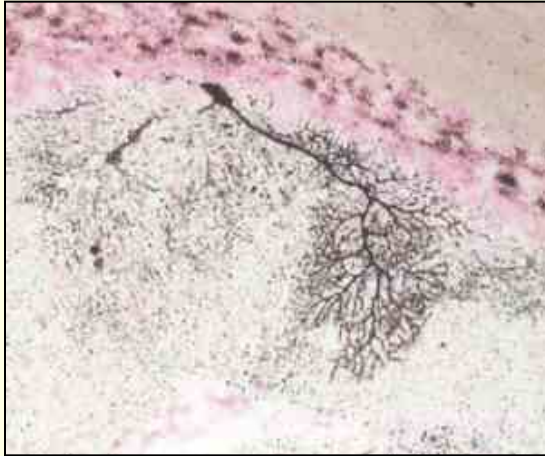
Disintegration  
Stain





# AREA Advantage: The additional “footprint” of 4 elements vs. 1 element makes assessment easier

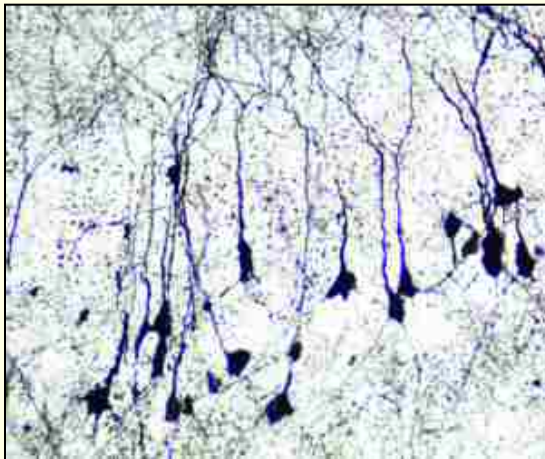
Dendrites



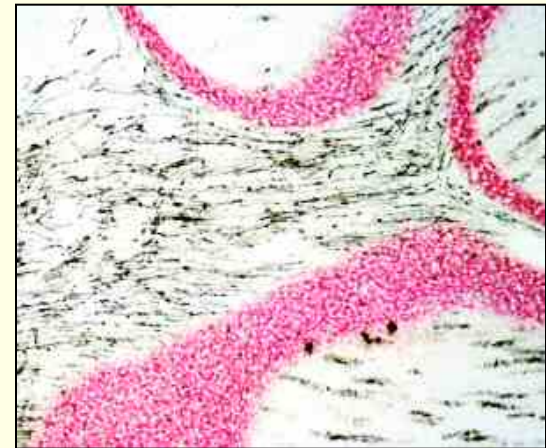
Synaptic terminals



Cell bodies



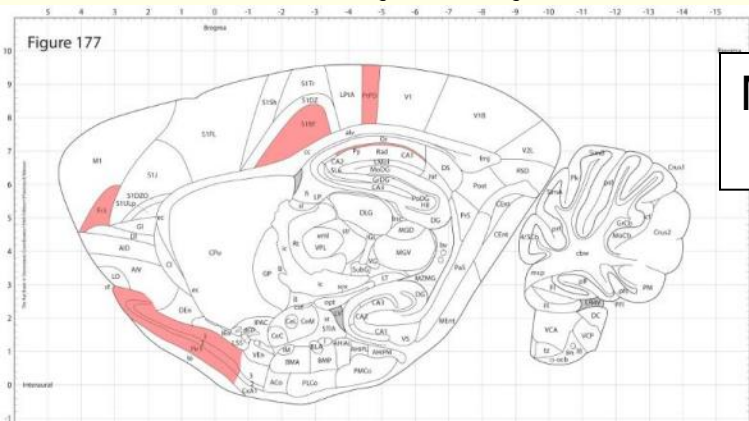
Axons



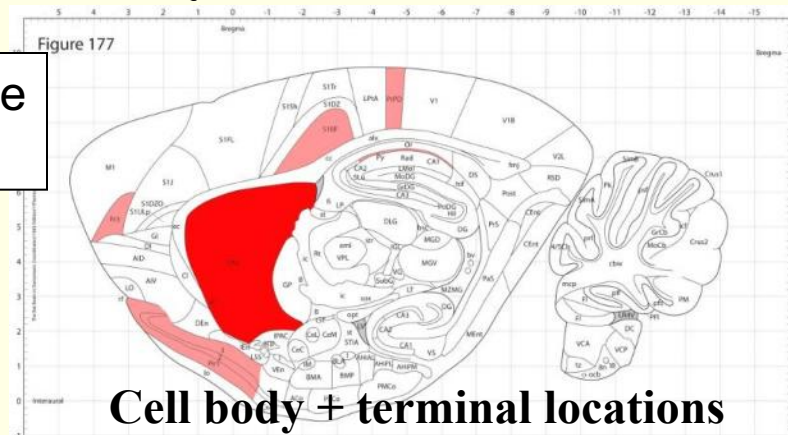
# The extended neuronal elements can often be observed in locations beyond that of cell bodies

## Cell body only locations

## Cell body + other elements

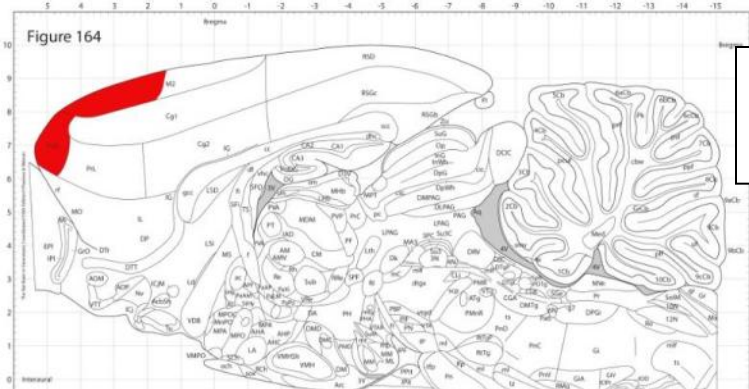


Methamphetamine



Cell body + terminal locations

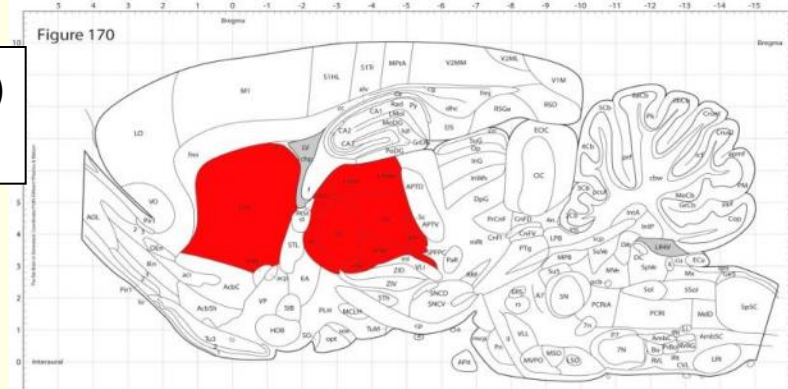
Other areas in which neuronal cell death can be observed (not seen in this section): indusium griseum, tenia tecta, fasciola cinerea



MDMA (Ecstasy)



Cell body (as well as axonal and terminal) staining can be seen in fronto-parietal cortex

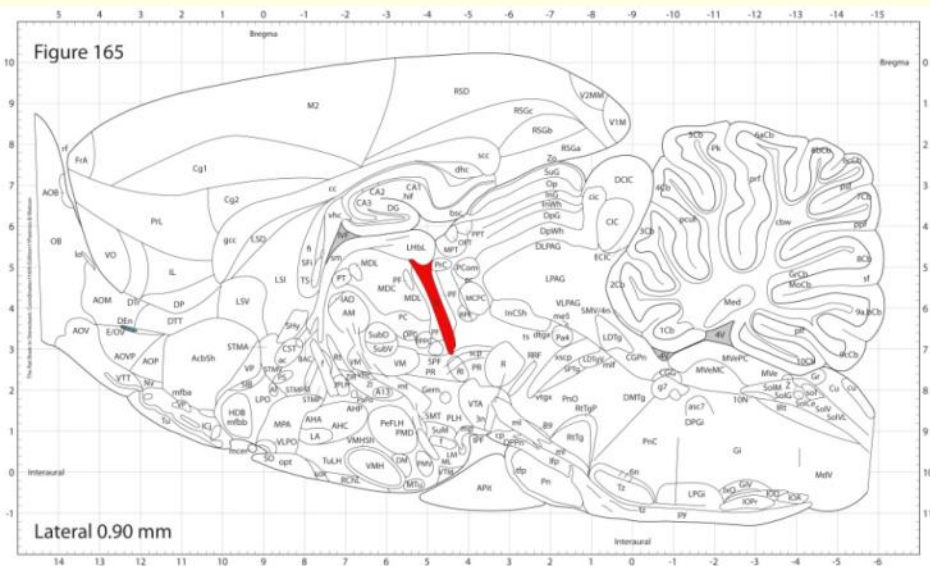


Terminals are stained throughout striatum and both axons and terminals can be observed in the thalamus

***A more comprehensive scope of damage is achieved when all elements are considered in evaluations***

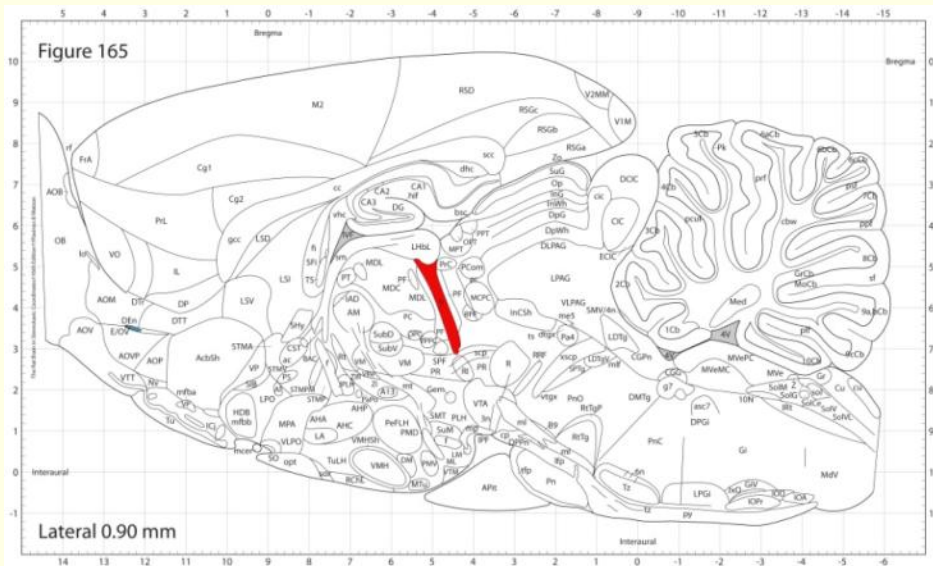
# Some compounds have only been observed to destroy elements other than the cell body

## Cocaine



**Cocaine** only destroys axons in the fasciculus retroflexus. Axons begin in the lateral habenula and travel ventrally in FR until they disperse in ventral mesencephalon.

## Nicotine

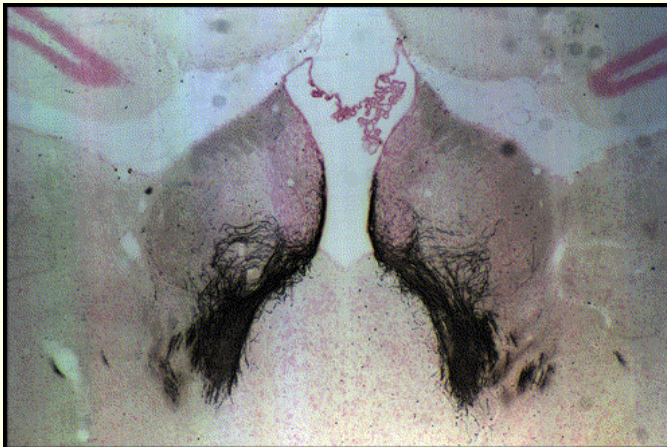
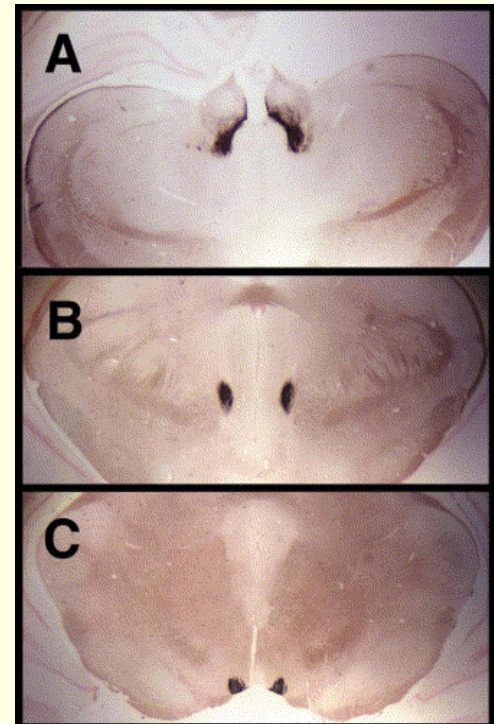
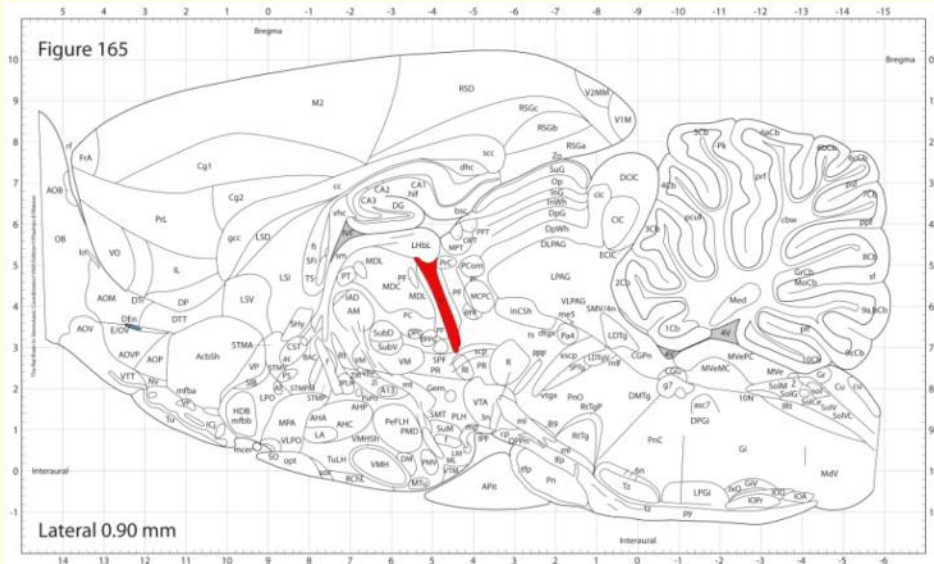


**Nicotine** destroys the axons in the cholinergic sector of the FR, which runs from the medial habenula through the core of FR to the interpeduncular nucleus.

*Even in the absence of cell body death, the neuron is incapacitated*



# Axonal degeneration from nicotine



# Primary Study Design Essentials for Baseline Evaluations (Acute cell death)

- ▶ Timing:
  - ▶ Sampling at 48h and 96h after initial dosing would detect all known compounds causing acute cell death
  - ▶ Sampling at 72hr after dosing detects MOST compounds
- ▶ Location:
  - ▶ Sampling at 50-60 levels virtually guarantees representation of all regions of the brain
  - ▶ Sampling at 20+ levels wouldn't ensure full evaluation of all regions but MOST would be available for evaluation
- ▶ Stains:
  - ▶ Use of a degeneration specialty stain allows most accuracy and efficiency in analysis (CuAg and FluoroJade methods)
  - ▶ Cellular markers (H&E, Nissl, TUNEL) and apoptosis markers (Caspase) are candidate markers but far less suitable than degeneration specialty stains

## Modifiers to baseline testing:

- ▶ Repetitive dosing considerations
- ▶ Developmental neurotoxicity considerations

# **REPETITIVE DOSING: SUB-CHRONIC AND CHRONIC CONSIDERATIONS**

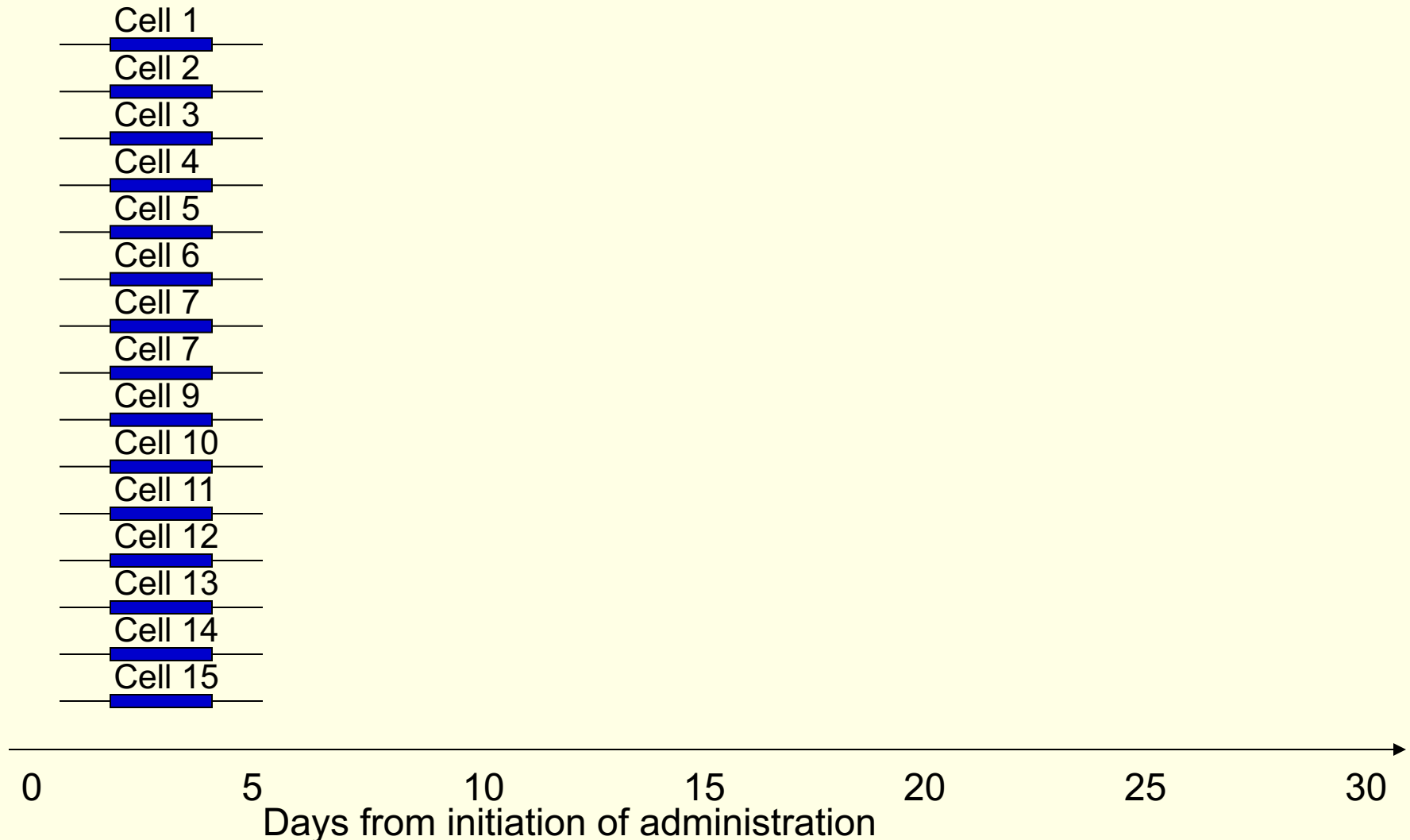
# Acute, Subchronic and Chronic studies require varied approaches in neurotoxicity assessments

- ▶ Experientially, (including environmental and other compounds) over 80% of neurotoxic compounds cause their observable damage during the acute time period (1-10 days)

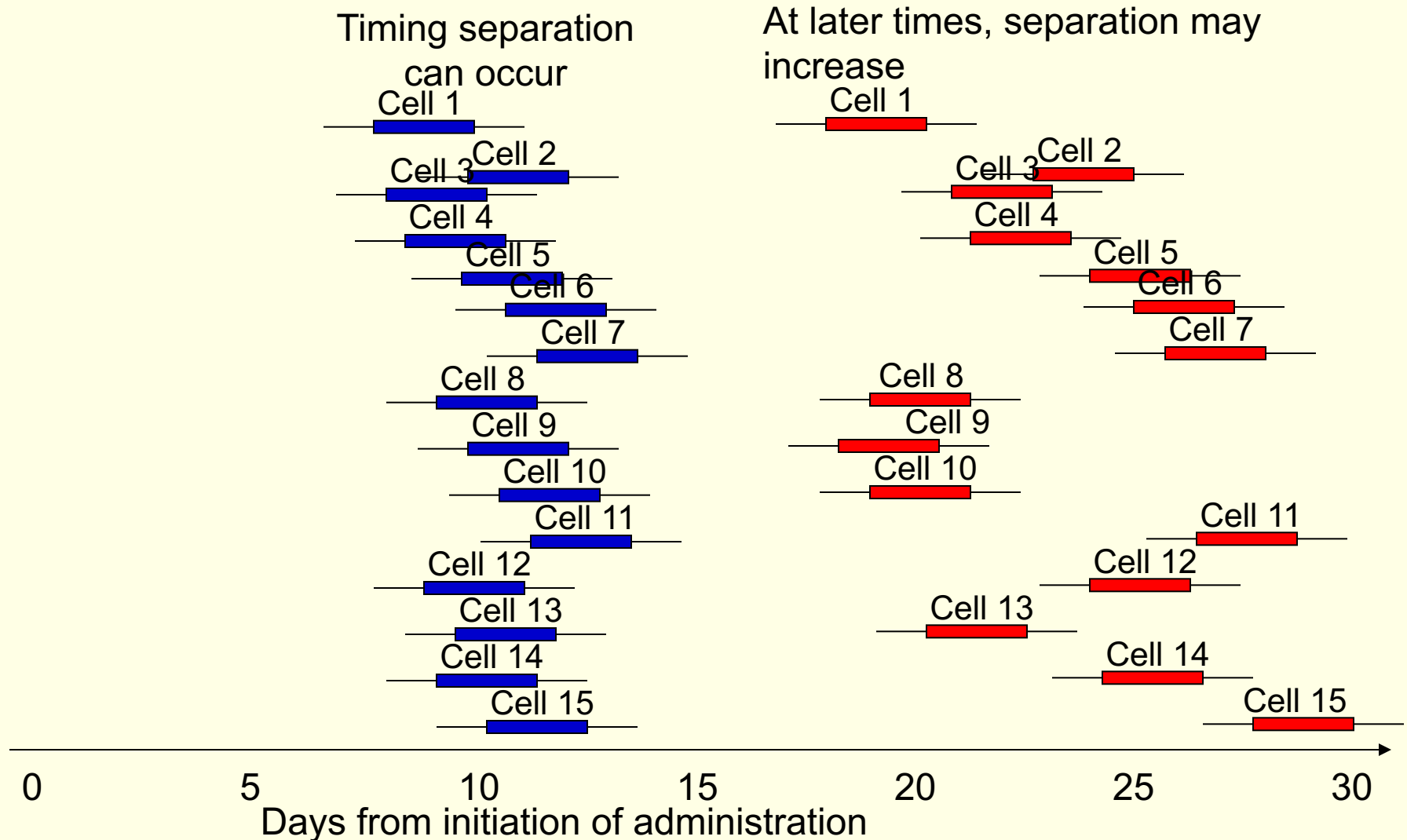
*The temporal attributes of cell death are more varied in subchronic and chronic time-frames (vs. acute), however many of the same principles can be adapted*



# In acute cell death, vulnerable cells die in a simultaneous pattern



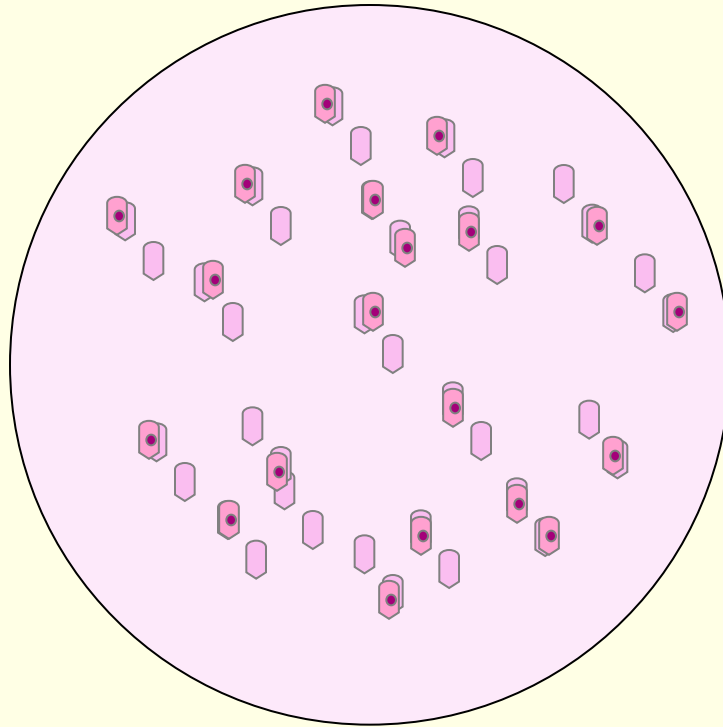
With chronic and subchronic cell death, vulnerable cells have the potential to die in a staggered pattern



***Timing patterns for subchronic and chronic cell death are not as well understood***

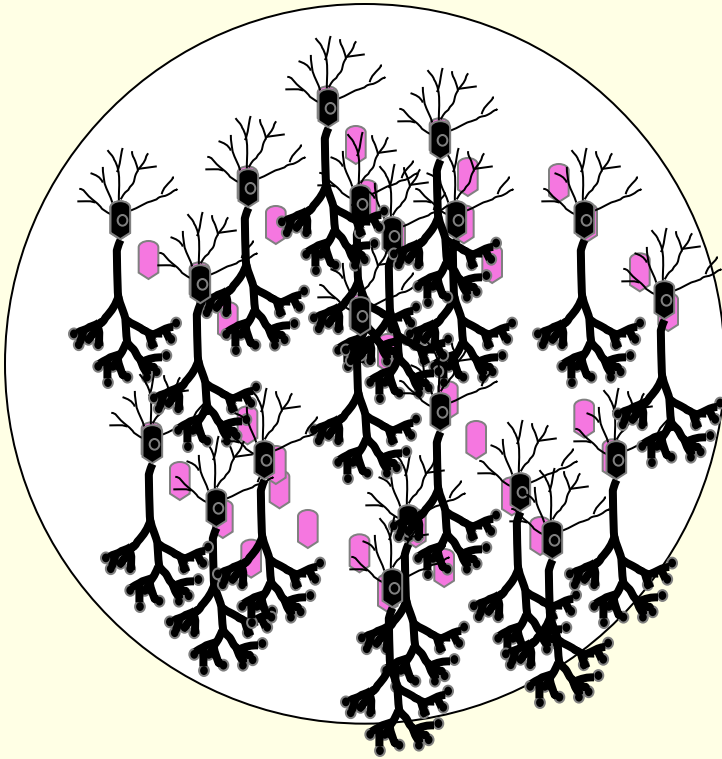
# Fewer cells can be witnessed dying at any point in time in subchronic and chronic cell death

Time lapse over weeks, months or years

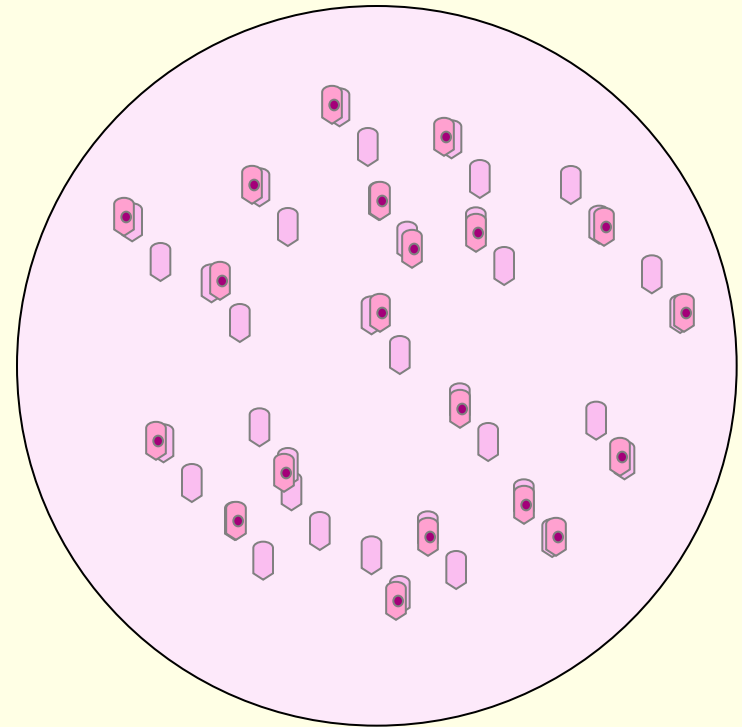


***Although little damage is observable at any point in time, the cumulative effect is comparable to what was demonstrated for acute response***

An increased footprint is an advantage when cell death events are spread out in subchronic and chronic studies



Footprint of all elements



Footprint of cell body (only)

## Subchronic and chronic effects are more difficult to detect

- ▶ The signal of cell death is likely to be very “light” (just a few cells) at any point in time
- ▶ Periodic intervals sacrifice time during a course of administration are still required to constitute a reasonably adequate observation.
- ▶ Temporal sampling in Subchronic and Chronic study designs is a trade-off between thorough and practical



# Subchronic and Chronic Study Design Recommendations

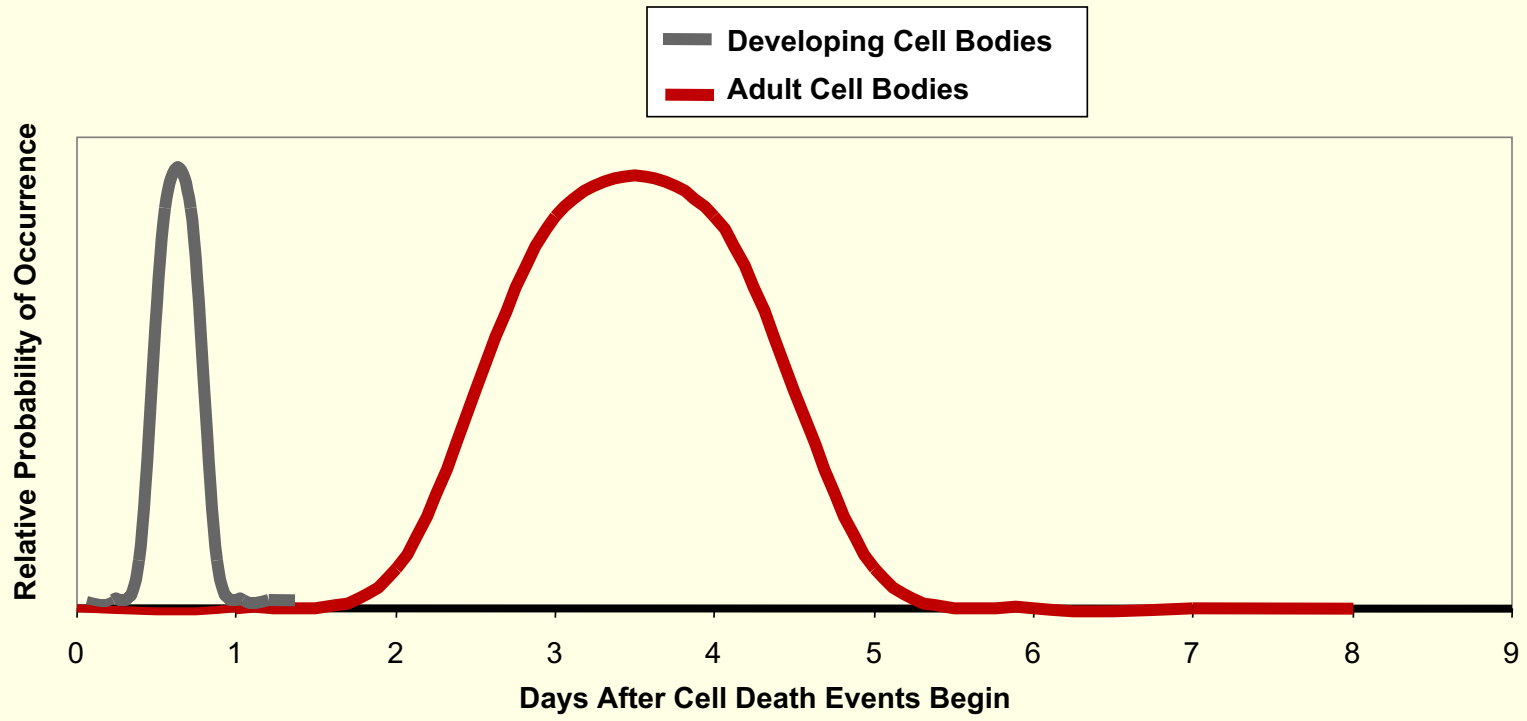
- ▶ Most important: These are add-ons to the baseline protocol details
- ▶ Subchronic sacrifice times:
  - ▶ 9-10 day, 16-20 day, 25-30 day
- ▶ Chronic sacrifice times:
  - ▶ From 30-90 days: monthly
  - ▶ From 90 days on: every 3 months

# Developmental Neurotoxicity

## Principles are the same but timelines are accelerated for cell death and clearance of debris

- ▶ In rodents, 48h and 96 h sampling recommendations become 12h and 24h from PND3-PND25
- ▶ Different cells are vulnerable at different development ages so if a range of ages is considered for therapy, all must be tested uniquely
- ▶ Caspase-3 or Caspase-9 IHC can be added as a secondary marker to look for apoptosis changes

In the developing brain the window of opportunity for measurable neurodegeneration is shrunk from days to hours



**VARIATIONS IN SCOPE:  
MOVING UP THE STEPWISE  
LADDER**



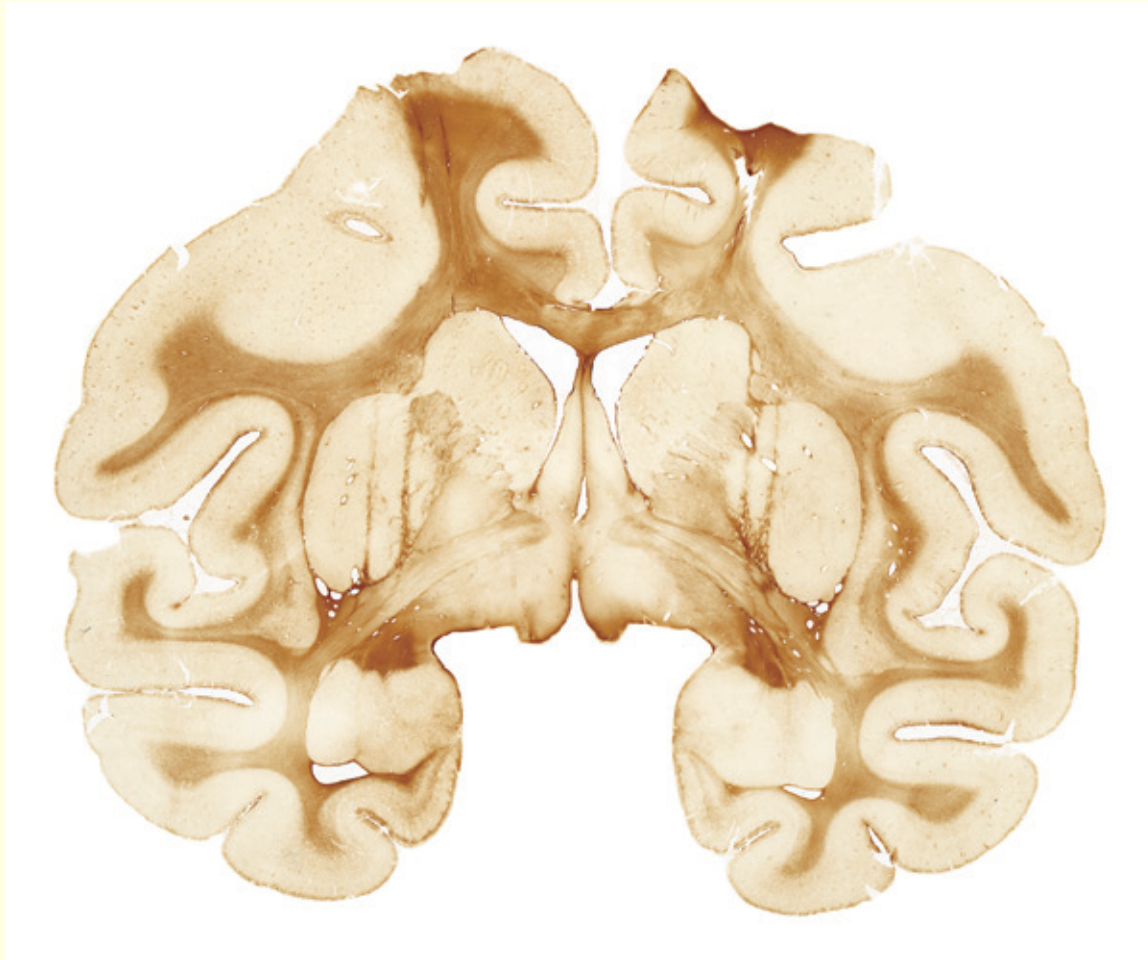
# Inflammation and Perturbation

- ▶ Depending on the specific therapy, the induction of any inflammation may be a concern
- ▶ Of greater concern is inflammation that persists over time or becomes worse

# Assessing for inflammation and perturbations

- ▶ Inflammation is effectively evaluated through reactive microglia (Iba1) and reactive Astrocytes (GFAP):
- ▶ Iba1 is most effective at revealing reactive microglia from ~7 days following dosing through several weeks after dosing
- ▶ GFAP detects astrocyte perturbation as earlier as 36 hours following insult, peaking at ~72 hours and persisting in detectable state for several weeks minimum

Evidence of inflammation can persist for a long time  
but should dissipate if the cause is removed



***It is clear that past needle tracts caused inflammatory response but that response has subsided and the evidence is confined to the tracts themselves***

# Inflammation protocols:

- ▶ Should be used when:
  - ▶ Any inflammatory response is considered unacceptable
  - ▶ Inflammatory response over time is being evaluated
- ▶ Design:
  - ▶ Timing: ~ 1 week after insult to view acute response. Weeks and months later to evaluate if reduced response
  - ▶ Stain(s): GFAP and Iba1

# NMDA Receptor Antagonists

- ▶ This class of compounds follows classic degeneration profiles.
- ▶ Baseline testing followed by relevant additions for subchronic and chronic exposure are perfectly suitable for these evaluations
- ▶ It is not necessary to look for vacuoles or apoptosis: the baseline marker of cell death captures the final result of these independently

# NMDA Receptor protocols:

- ▶ Should be used when:
  - ▶ Any time an NMDA receptor therapy is being evaluated
- ▶ Design:
  - ▶ Standard Baseline Acute Cell Death Protocol
  - ▶ Modifier: Add developmental protocol sacrifice times if intended for juveniles
  - ▶ Modifier: Add subchronic and chronic protocol timepoints if administered more than once



# Cholinergic evaluations

- ▶ Drugs affecting Nerve Growth Factor (NGF) can affect the cholinergic cell population.
- ▶ Analysis of the status of the cholinergic population utilizes the enzyme involved with the synthesis of acetylcholine, Choline Acetyl Transferase (ChAT).
- ▶ ChAT positive neurons are widespread in the brain, but the target for analysis of for a number of would be drugs affecting the cholinergic system has been the nucleus of the diagonal band of Broca and Meynert's nucleus basal, both found in the ventral forebrain. Both structures project heavily to the neocortex. Meynert's nucleus has been found to be depleted in Alzheimer's disease.

# Cholinergic Evaluation protocols:

- ▶ Should be used when:
  - ▶ A therapy affecting Nerve Growth Factor (NGF) is used
- ▶ Design:
  - ▶ Use ChAT IHC to evaluate expression of ChAT
  - ▶ Evaluation areas should include diagonal band of Broca and Meynert's nucleus basal

## Surgical treatments

- ▶ Surgery and injections to spinal cords and brain will obviously create some baseline damage due to entering CNS tissues.
- ▶ Cell death and inflammation are to be expected

## Surgical treatments: Evaluation

- ▶ Baseline Acute Cell death protocol can be used to assess extent of normal damage caused by treatment
- ▶ Inflammation protocols can be used to assess early and persistent inflammation

# Stem Cell therapy

- ▶ Goal is to:
  - ▶ Confirm the cells survive
  - ▶ Confirm cells do not proliferate (become neoplastic)
  - ▶ Confirm cells remain in the target area without causing damage

## Recommended Efficiencies

- ▶ Minimize positive controls: should only be used to confirm a stain works, not for comparison
- ▶ Reduce overlapping stains (i.e. Degeneration, H&E, Activated Caspase all requested)
- ▶ Recommend and allow the use of specialized stains which lessen the burden on pathologists and improve accuracy of assessment



# PROTOCOLS SUMMARY

# Protocols Summary

## Compound-specific or function specific changes

Each is unique: Use endpoint specific marker in a timeline appropriate for expression

## INFLAMMATION/PERTURBATIONS

Standard: 7-10 days with GFAP and Iba1

(Modifier) Persistence: Compare inflammation signal at later time points

## BASELINE

Standard: 48h and 96h stained with CuAg or FluoroJade

(Modifier) Repetitive dosing: Add weekly assessments for 30 days, monthly for 30-90 days

(Modifier) Developmental: Acute evaluation becomes 12h and 24h until PND25

***All evaluations to be performed on 50-60 evenly spaced intervals ideally.  
20+ levels is bare minimum***

# FINAL THOUGHTS

There is far too much information to cover in this short window of time...  
this is an ever-evolving area and exciting to share contemporary principles  
of the toolkits of neurological safety testing with you

Thank you!

Q&A

## Further Discussion Topics

- ▶ Developmental neurotoxicity
- ▶ Spinal cord assessments
- ▶ Biomarker development potential
- ▶ Reduced need for controls with degeneration staining methods

# Appendix

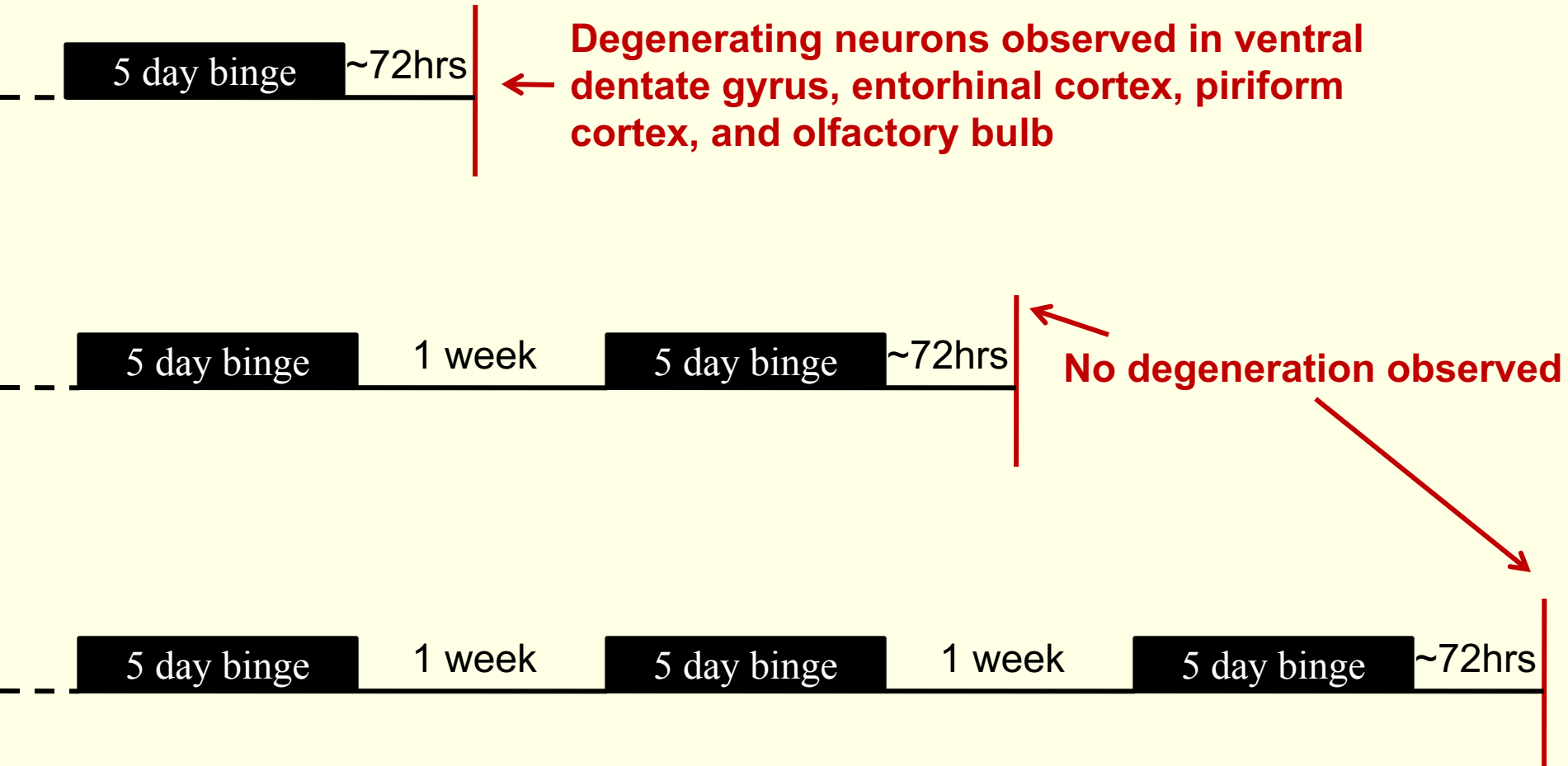


# Safety screening pitfalls in consideration of potential locations of effect

- ▶ Assessing the brain only in areas anticipated to be vulnerable to damage
- ▶ Sampling single levels from just the “popular” structures
- ▶ Sampling at excessive intervals

# Once vulnerable cells die, subsequent administration of a compound may not induce further cell death

## Case Study: Alcohol



*In this study, all susceptible cells died during the first exposure period*

# Classic Acute Neurotoxicity Example: MK-801

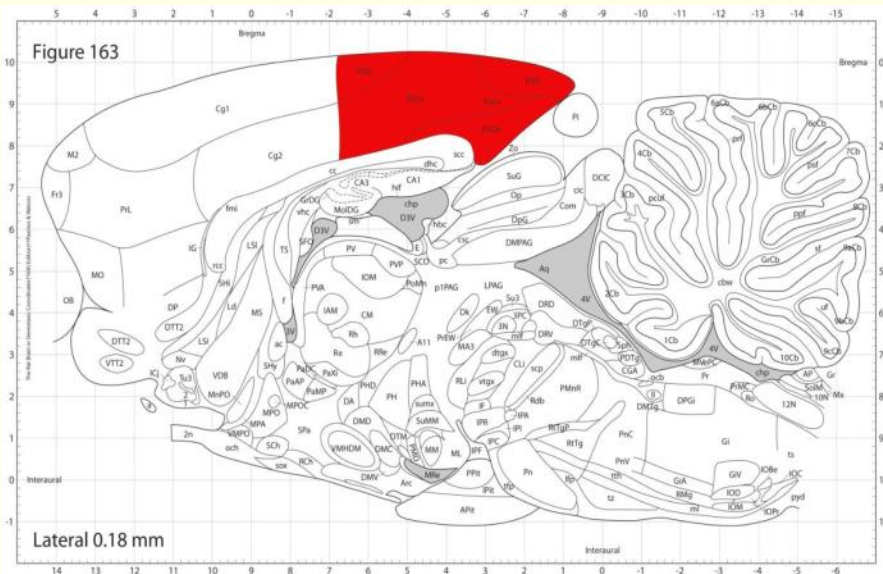
## The history and profile of MK-801 highlights many of the principles outlined as fundamentals to neurotoxicity

- ▶ MK-801 is an excellent NMDA receptor antagonist and was a promising therapeutic candidate
  - ▶ Still used as a benchmark today
- ▶ In 1989 John Olney observed intracytoplasmic vacuoles in rat brains following MK-801 administration
- ▶ These vacuoles were observed to be transient
- ▶ The vacuoles are commonly referred to as “Olney lesions”

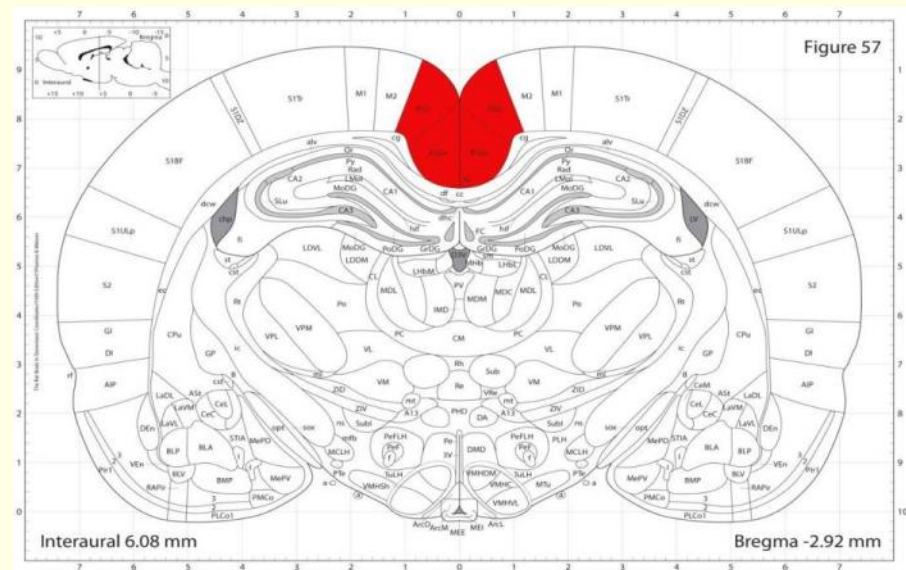
***The presence of these vacuoles was appropriately the source of much concern and debate about the risk of MK-801***

# Intracytoplasmic vacuoles occur in the posterior cingulate/retrosplenial cortex in response to MK-801

## Sagittal section of retrosplenial cortex

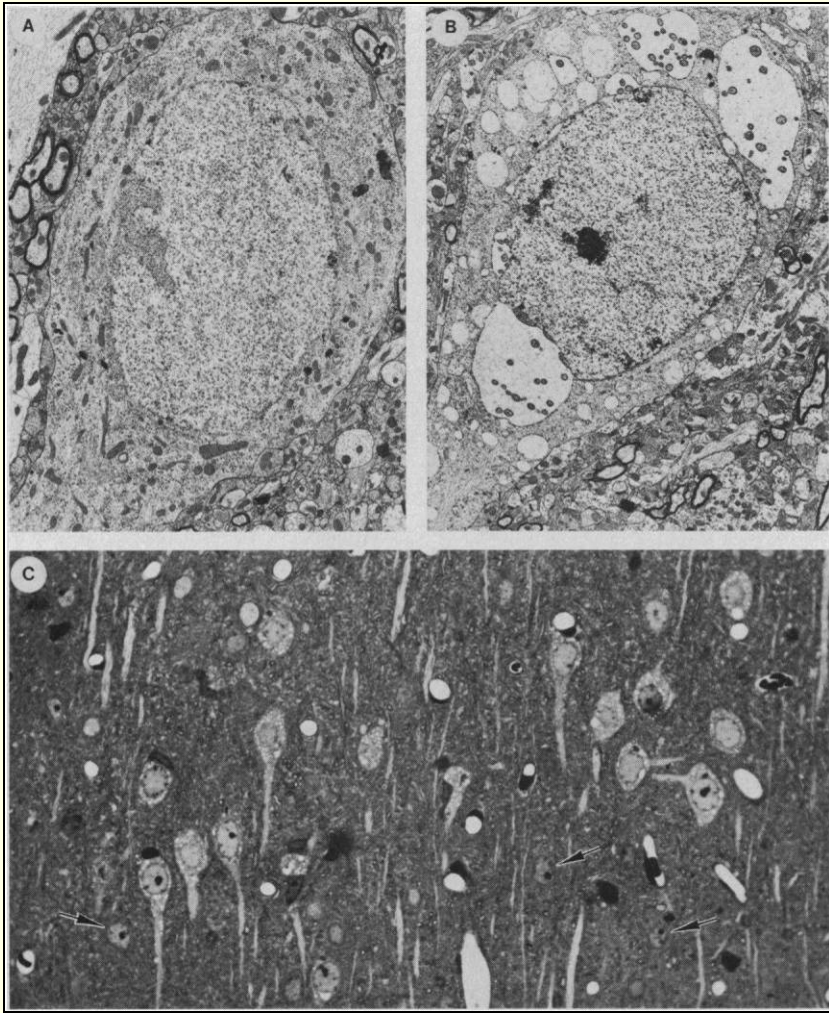


## Coronal section of retrosplenial cortex

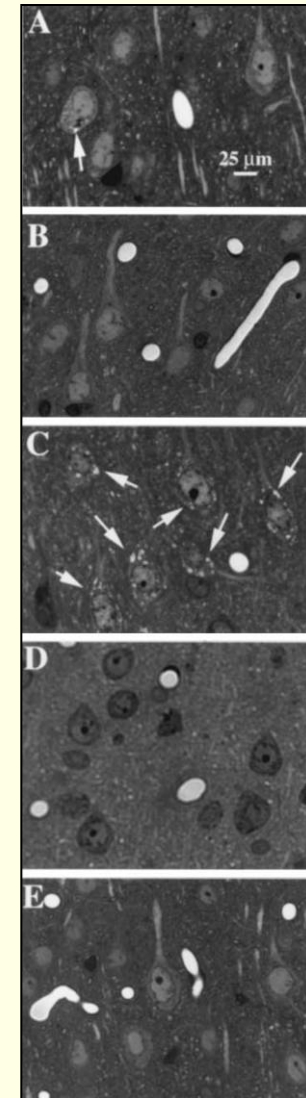


Maas, Indacochea, Muglia, Tran, Vogt, West, Benz, Shute, Holtzman, Mennerick, **Olney**, Muglia (2005)  
*Journal of Neuroscience*

# The Olney lesions can be observed using the Toluidine blue method



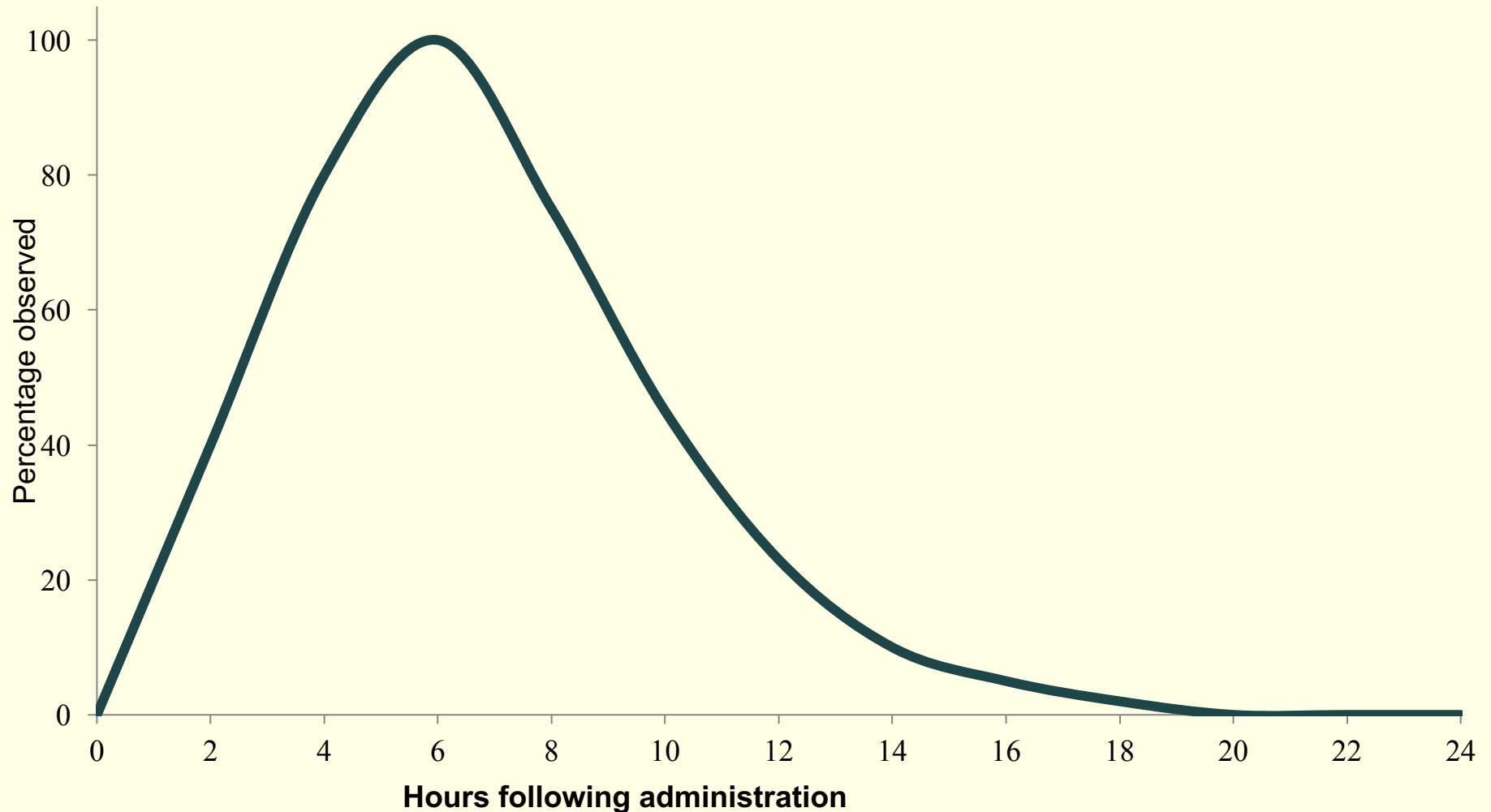
Olney, Labruyere, Price (1989) *Science*



Jevtovic-Todorovic, Benshoff, Olney (2000) *British Journal of Pharmacology*



# Vacuoles can be seen from 2-12 hours after MK-801 administration and peak 4-6 hours



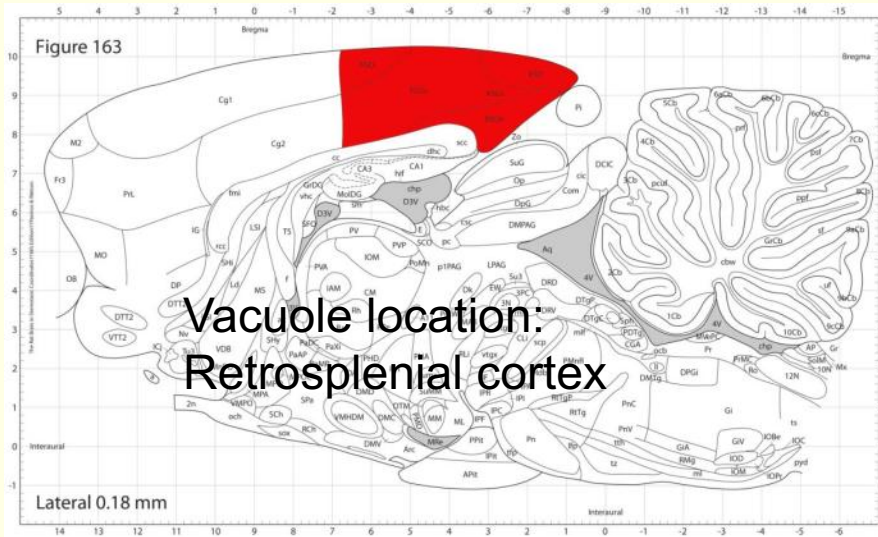
Evidence of permanent damage from MK-801 was confirmed when neuronal degeneration was observed

- ▶ Olney and others published in 1990 and 1993 that MK-801 caused neuronal degeneration.
- ▶ This neurodegeneration was found co-located at vacuole sites...
- ▶ Importantly, neurodegeneration was also found in regions of the brain distant from the vacuole sites.

*The finding of neurodegeneration was significant both in its indication of permanent damage and as a reminder that location of effects can be unpredictable.*

MK-801 causes cell death in numerous structures other than retrosplenial cortex

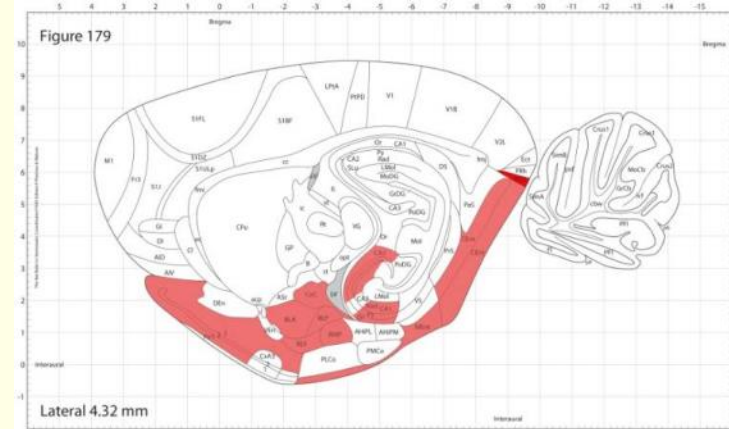
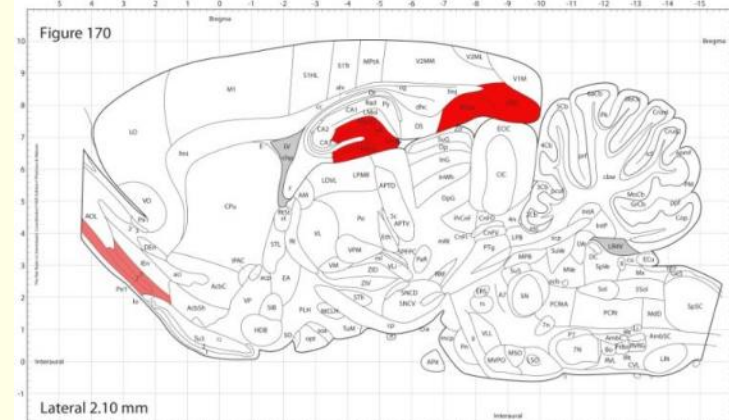
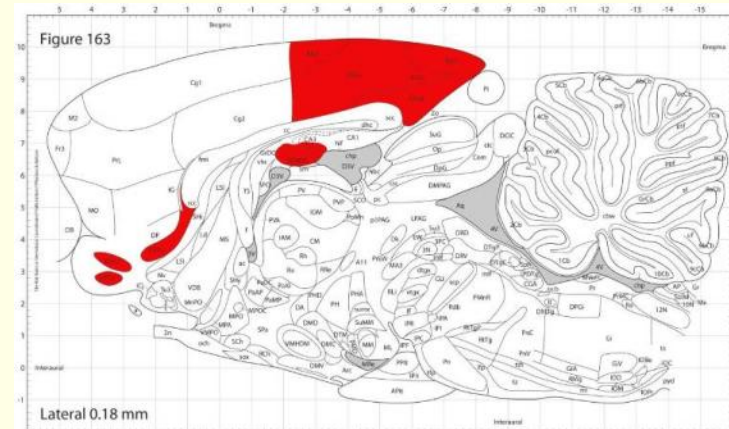
## Cell Death Locations



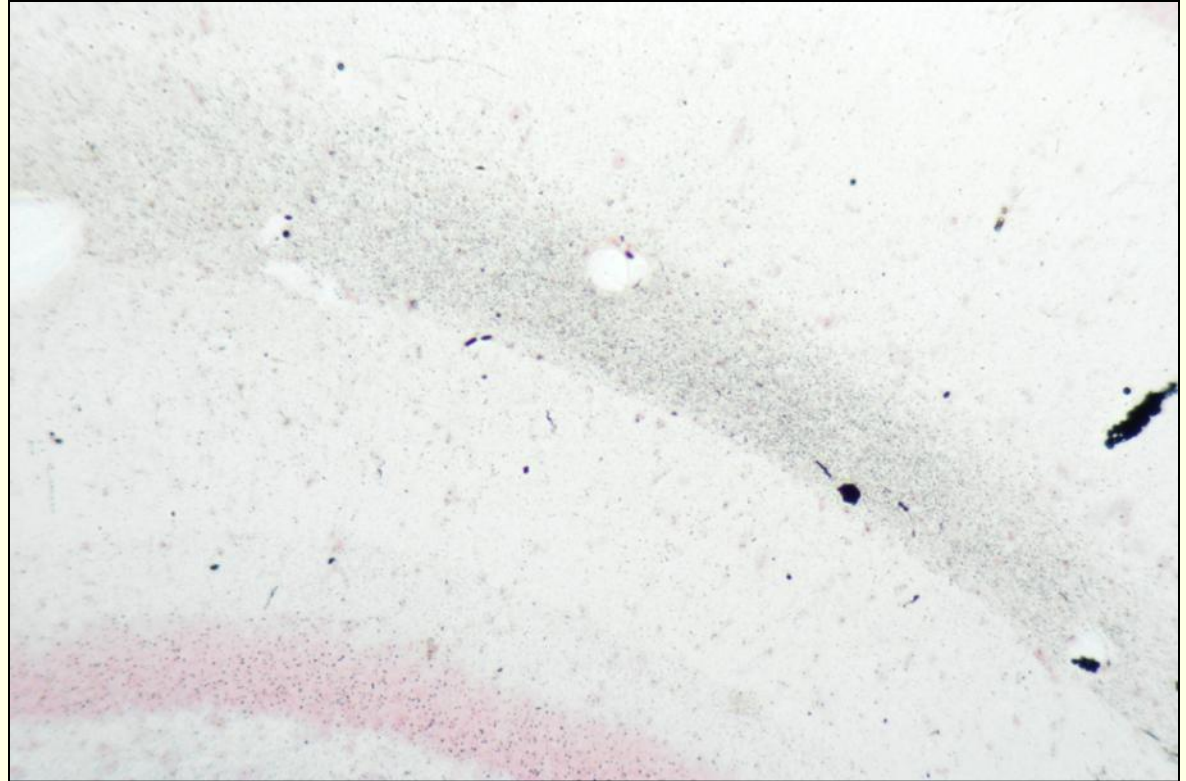
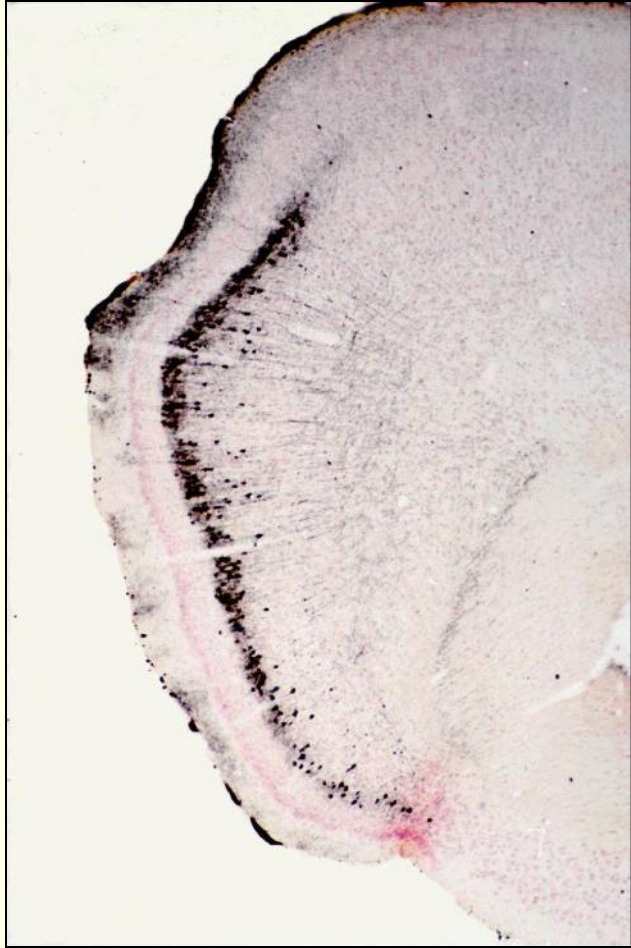
Horvath, Czopf, Buzsaki (1997) *Brain Research*

MK-801 destroys cells in:

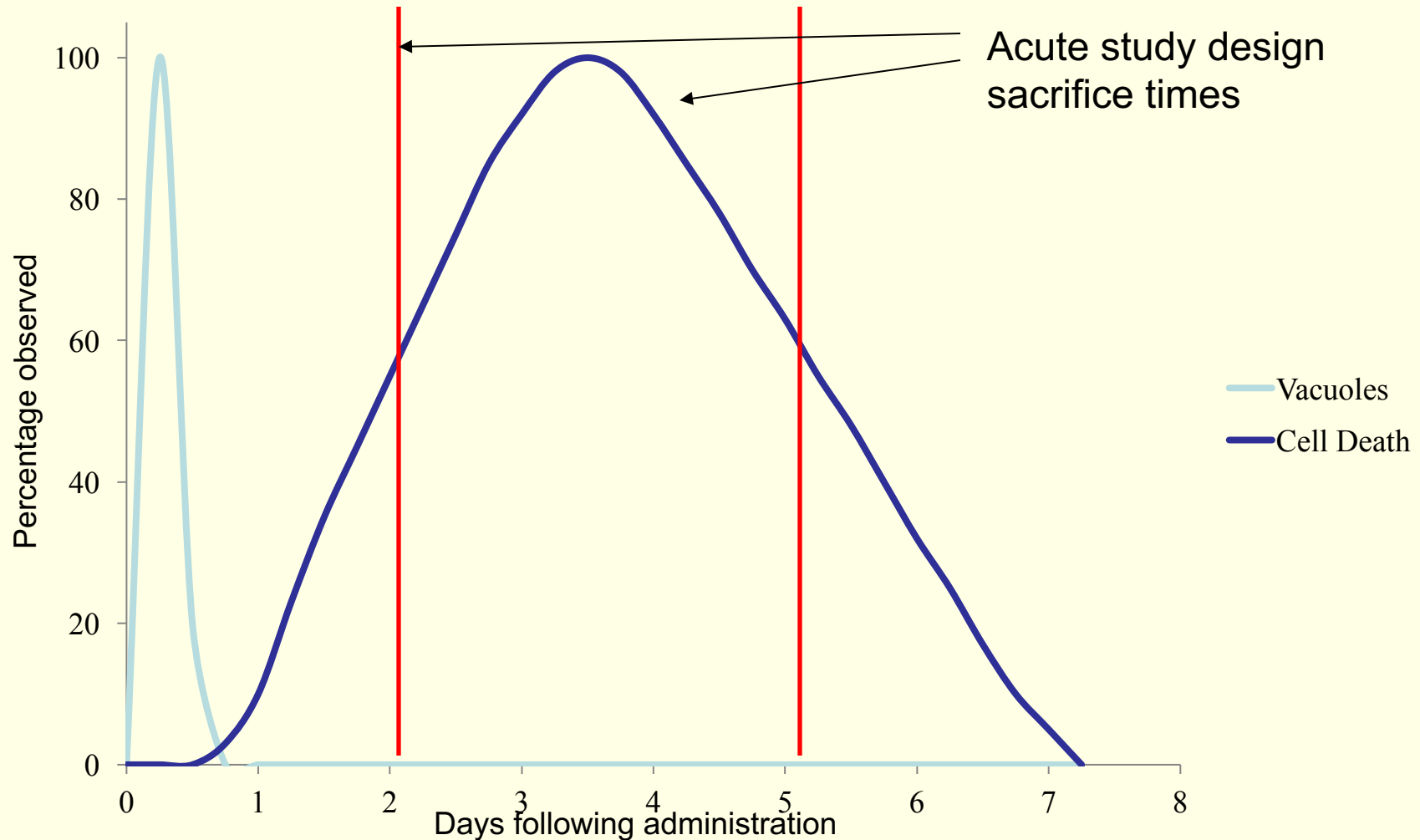
- Retrosplenial cortex
  - Tenia tecta
  - Dentate gyrus
  - Pyriform cortex
  - Amygdala
  - Entorhinal cortex
- Ventral CA1 and CA3 of hippocampus



# MK-801 degeneration images



The peak observable time of degeneration following administration of MK-801 lasts ~ 3 days.



***The cell death pattern for MK-801 is a classic example of an acute neurodegeneration pattern***

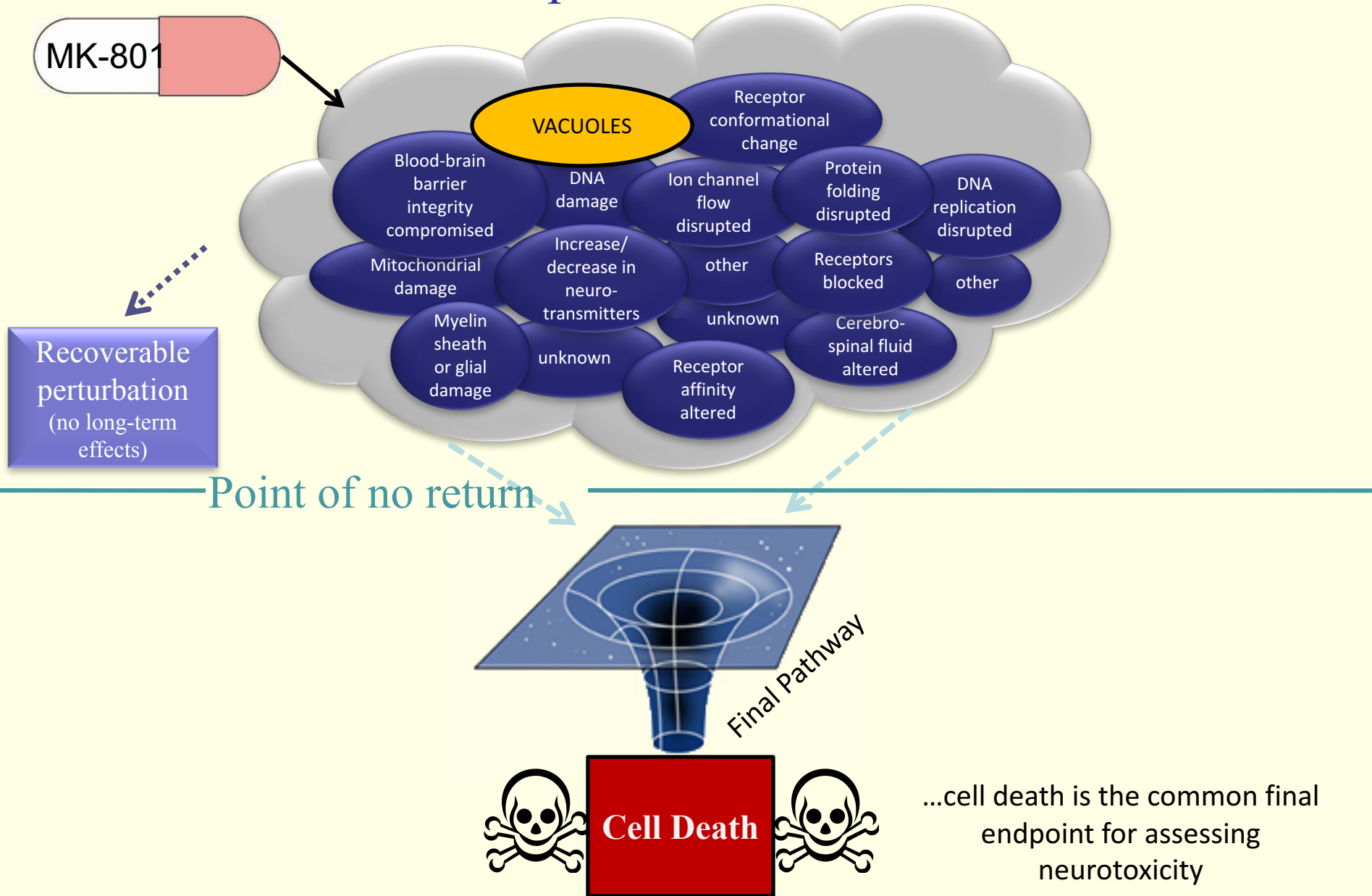
# MK-801 has remained a heavily studied compound

- ▶ During the time following the initial finding of neurodegeneration research on MK-801 has continued:
  - ▶ The mechanics of the MK-801 reaction have been studied and documented extensively
  - ▶ The relationship between the vacuoles and degeneration has been probed
  - ▶ Degenerating elements have become the accepted single indicator of irreversible damage

***Although significant and unique, the initial observation of vacuoles is more important as a sequence of events, rather than as an endpoint***



# Vacuoles are one of many potentially recoverable events that often precede cell death



# The MK-801 example is a case study that highlights many of the principles of neuropathologic assessment

- ▶ Location lessons:
  - ▶ Assess throughout the brain
  - ▶ Assess in areas where effects are unexpected
- ▶ Timing examples:
  - ▶ Neurodegeneration most often occurs as a direct, acute response
  - ▶ Assessment at multiple time points maximizes observation potential
- ▶ Scope considerations:
  - ▶ All of the neuronal elements contribute to the footprint of detection

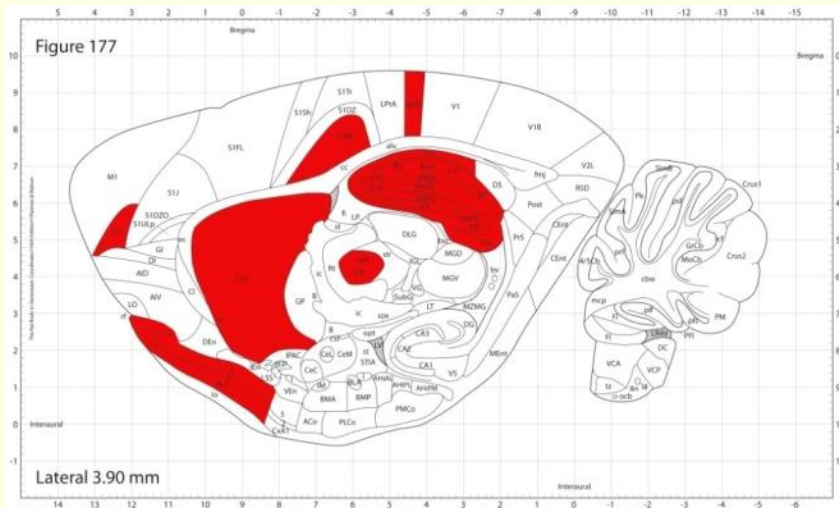
***The routine neuropathologic study design based on contemporary science is designed to reveal permanent damage***

# When and where is the brain affected by neurotoxins?

Neurotoxin	Time point in days	Location at peak cell death
Alcohol	3	olfactory bulb, posterior pyriform, entorhinal cortex, dentate gyrus
Amphetamine	3	parietal cortex, barrel field of primary somatosensory cortex, frontal cortex, hippocampus, tenia tecta, piriform cortex, septum, caudate putamen, thalamic nuclei (PV, CM PC/CI, VM/VL, VPL)
Domoic acid	3	olfactory bulb, anterior olfactory nucleus, dorsal tenia tecta, lateral septal nucleus, reuniens thalamic nuclei, hippocampus (pyramidal cell layer), amygdalohippocampal area
Kainic acid	0.5-3	CA1, CA3, polymorphic layer of dentate gyrus, parasubiculum
Methamphetamine	3	parietal cortex, barrel field of primary somatosensory cortex
MDMA	.75-3	frontoparietal region of neocortex
MK-801	1-4	retrosplenial cortex; dentate gyrus; pyriform cortex; tenia tecta; amygdala; entorhinal cortex
MPTP	2-2.5	VTA; substantia nigra
3-nitropropionic acid (3NPA)	2.5	caudate putamen, prefrontal cortex, insular cortex, entorhinal cortex, parietal and sensory cortex, CA1, CA3 and dentate gyrus of hippocampus
2'-NH <sub>2</sub> -MPTP	2-2.5	dorsal raphe
p-chloroamphetamine (PCA); low dose	1-3	raphe nuclei (B-7 and B-8), B-9 serotonergic cell group, ventral midbrain tegmentum;
PCP HCl (phencyclidine)	1	entorhinal cortex, dentate gyrus in ventral hipp, cingulate and retrosplenial cortex

Varied neurotoxins produce cell death in differing locations in the brain

# Acute Neurodegeneration Profile for Amphetamine

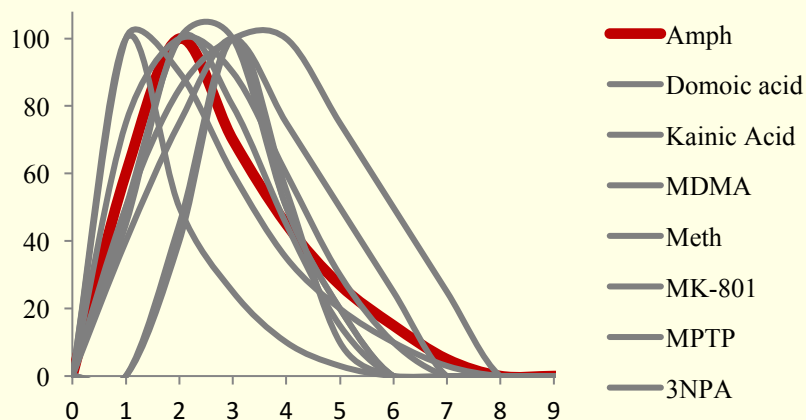


## ► Location

- Parietal cortex
- Barrel field of primary somatosensory cortex,
- Frontal cortex
- Hippocampus
- Tenia tecta (*not shown in this section*)
- Piriform cortex (*not shown in this section*)
- Septum
- Caudate putamen
- Thalamic nuclei (PV, CM PC/CI, VM/VL, VPL)

## ► Timing

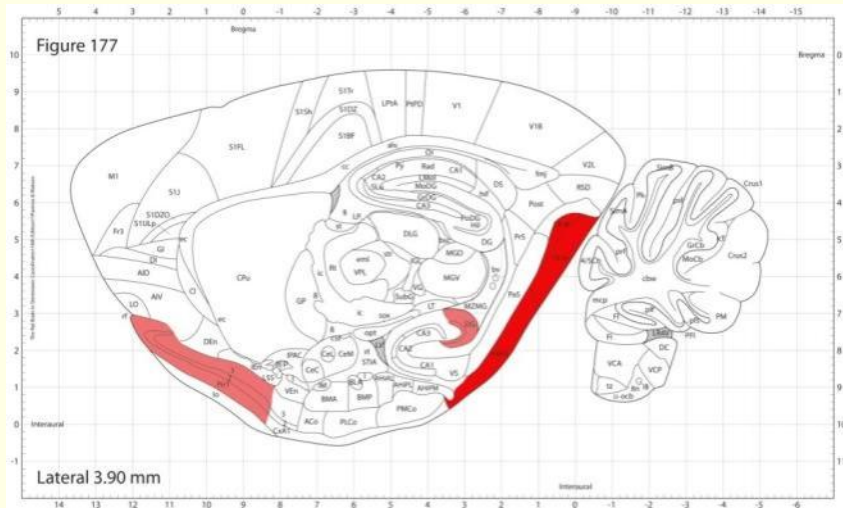
- 1 day after dosing: neurodegeneration-labeled cells were seen
- 2-3 days after dosing: peak neurodegeneration labeling
- 4 days after dosing: significant decreases in neurodegeneration-labeled cells
- 14 days post-administration: neurodegeneration was barely detectable



# Acute Neurodegeneration Profile for Amphetamine

- ▶ Belcher, A.M., S.J. O'Dell, and J.F. Marshall, *Impaired Object Recognition Memory Following Methamphetamine, but not p-Chloroamphetamine- or d-Amphetamine-Induced Neurotoxicity*. Neuropsychopharmacology, 2005. **30**(11): p. 2026-2034.
- ▶ Bowyer, J.F., et al., *Neuronal degeneration in rat forebrain resulting from -amphetamine-induced convulsions is dependent on seizure severity and age*. Brain Research, 1998. **809**(1): p. 77-90.
- ▶ Bowyer, J.F., R.R. DeLongchamp, and R.L. Jakab, *Glutamate N-methyl-D-aspartate and dopamine receptors have contrasting effects on the limbic versus the somatosensory cortex with respect to amphetamine-induced neurodegeneration*. Brain Research, 2004. **1030**(2): p. 234-246.
- ▶ Bowyer, J.F., *Neuronal degeneration in the limbic system of weanling rats exposed to saline, hyperthermia or d-amphetamine*. Brain Research, 2000. **885**(2): p. 166-171.
- ▶ Carlson, J., et al., *Selective neurotoxic effects of nicotine on axons in fasciculus retroflexus further support evidence that this a weak link in brain across multiple drugs of abuse*. Neuropharmacology, 2000. **39**(13): p. 2792-2798.
- ▶ Ellison, G., *Neural degeneration following chronic stimulant abuse reveals a weak link in brain, fasciculus retroflexus, implying the loss of forebrain control circuitry*. European Neuropsychopharmacology, 2002. **12**: p. 287-297.
- ▶ Jakab, R.L. and J.F. Bowyer, *Parvalbumin neuron circuits and microglia in three dopamine-poor cortical regions remain sensitive to amphetamine exposure in the absence of hyperthermia, seizure and stroke*. Brain Research, 2002. **958**(1): p. 52-69.
- ▶ Jakab, R.L. and J.F. Bowyer. *The injured neuron/phagocytic microglia ration "R" reveals the progression and sequence of neurodegeneration*. in *Toxicological Sciences*. 2003: Society of Toxicology.

# Acute Neurodegeneration Profile for Alcohol



- ▶ Location:
  - ▶ Olfactory bulb
  - ▶ Posterior pyriform
  - ▶ Entorhinal cortex
  - ▶ Dentate gyrus
- ▶ Timing
  - ▶ After 4 infusions per day for 4 days
    - 1hr after last dose: greatest measurable damage
    - 16hrs after last dose: slightly less damage observed than first time point
    - 72hrs after last dose: slightly less damage observed than first time point
    - 168hrs after last dose: no remaining detectable damage
  - ▶ This indicates that the peak cell death was occurring 2-3 days after the first administration

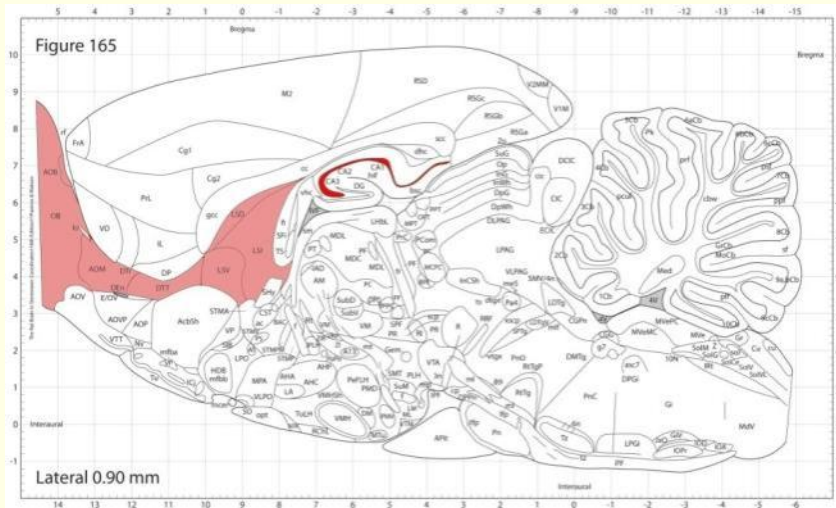
•Crews, F.T., et al., *Binge ethanol consumption causes differential brain damage in young adolescent rats compared with adult rats*. Alcoholism: Clinical and Experimental Research, 2000. **24**(11): p. 1712-1723.

•Han, J.Y., et al., *Ethanol induces cell death by activating caspase-3 in the rat cerebral cortex*. Molecules and Cells, 2005. **20**(2): p. 189-195.

•Ikegami, Y., et al., *Increased TUNEL positive cells in human alcoholic brains*. Neuroscience Letters, 2003. **349**: p. 201-205.

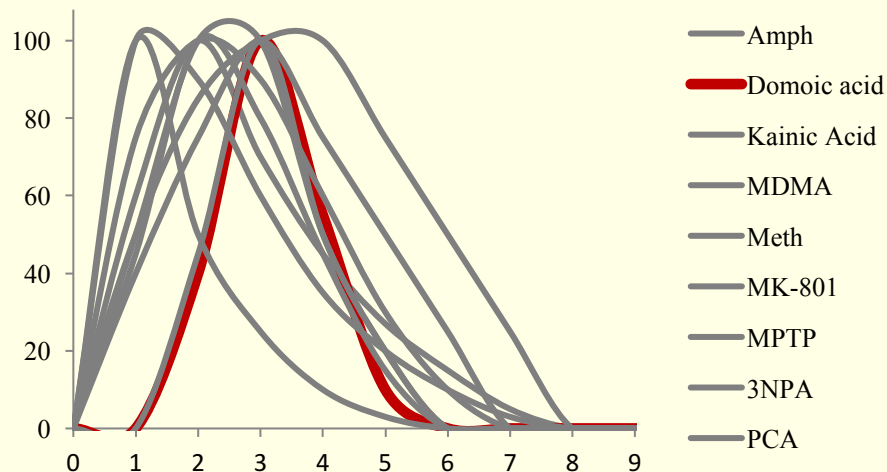


# Acute Neurodegeneration Profile for Domoic Acid



## ► Location:

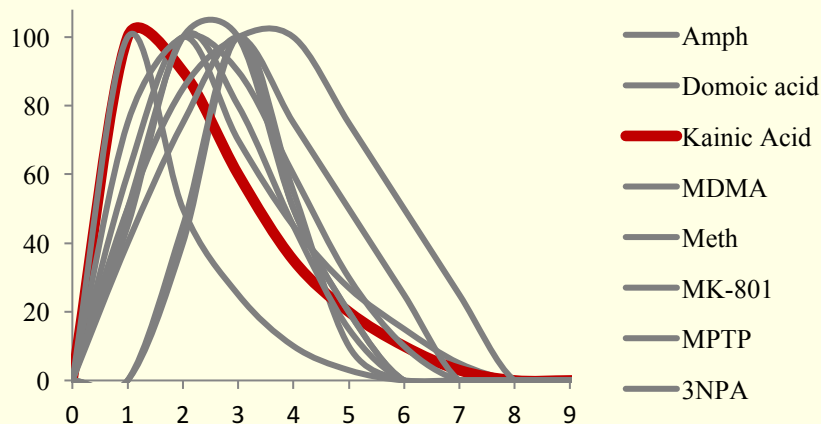
- Olfactory bulb
- Anterior olfactory nucleus
- Dorsal tenia tecta
- Lateral septal nucleus (*not shown at this level*)
- Reuniens thalamic nuclei
- Hippocampus (pyramidal cell layer)
- Amygdalohippocampal area (*not shown at this level*)



## ► Timing

- 3 days post-administration: labeling of cell bodies, synaptic terminals and axons were seen in many regions of the brain (low proportion of dendritic staining indicates that this was the peak time of cell death)

# Acute Neurodegeneration Profile for Kainic Acid



## ► Location:

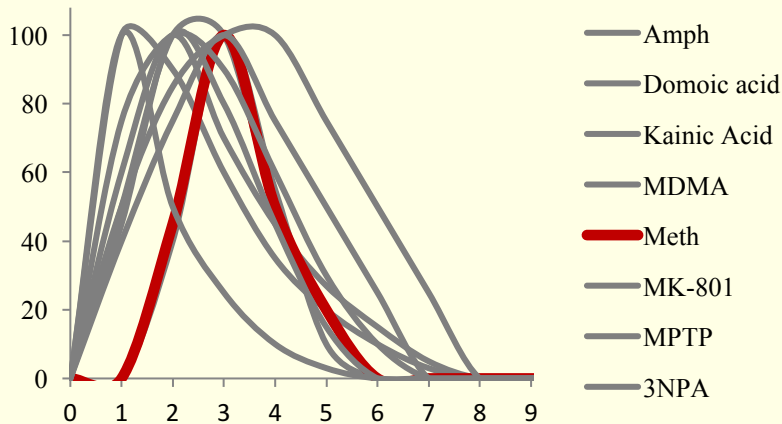
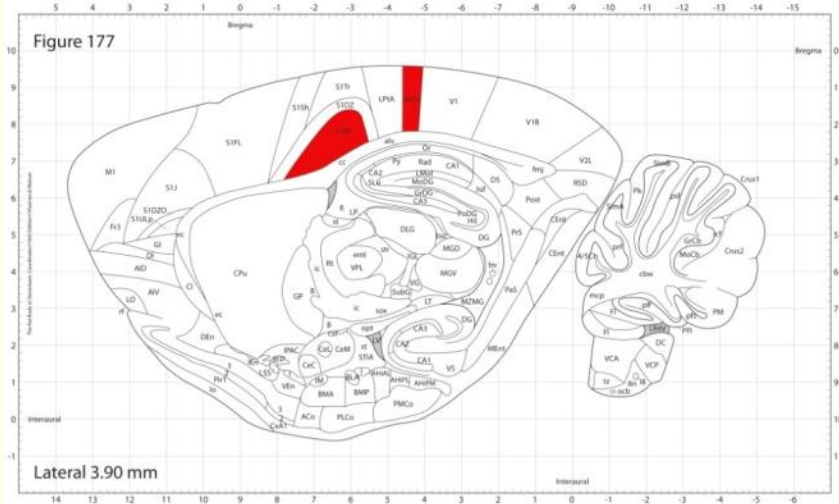
- Hippocampus (CA1, CA3)
- Dentate gyrus (polymorphic layer)
- Parasubiculum
- Entorhinal cortex

## ► Timing:

- 12hrs post-administration: scattered labeling
- 24hrs post-administration: heavy degeneration labeling in all areas listed
- 3 days post-administration: slightly diminished degeneration labeling in all areas listed
- 7 days post-administration: only one animal was observed to have residual degeneration
- 21 days post-administration: no observable degeneration

- Benkovic, S.A., J.P. O'Callaghan, and D.B. Miller, *Sensitive indicators of injury reveal hippocampal damage in C57BL/6J mice treated with kainic acid in the absence of tonic-clonic seizures*. Brain Research, 2004. **1024**(1-2): p. 59-76.
- Benkovic, S.A., J.P. O'Callaghan, and D.B. Miller, *Regional neuropathology following kainic acid intoxication in adult and aged C57BL/6J mice*. Brain Research, 2006. **1070**: p. 215-231.

# Acute Neurodegeneration Profile for Methamphetamine



- ▶ Location:
  - ▶ Cell bodies
    - Parietal cortex
    - Barrel field of primary somatosensory cortex
  - ▶ Axons and terminals (*not shown in this image*)

- Indusium griseum
- Tenia tecta
- Fasciola cinerea
- Pyriiform cortex
- Striatum (caudate-putamen)
- Cerebellum
- Fasciculus retroflexus

- ▶ **Timing**
  - ▶ 36-48hrs: neurodegeneration of axons and terminals observed
  - ▶ 3 days post-administration: neurodegeneration of cell bodies observed

- Belcher, A.M., S.J. O'Dell, and J.F. Marshall, *Impaired Object Recognition Memory Following Methamphetamine, but not p-Chloroamphetamine- or d-Amphetamine-Induced Neurotoxicity*. Neuropsychopharmacology, 2005. **30**(11): p. 2026-2034.
- Ellison, G., *Neural degeneration following chronic stimulant abuse reveals a weak link in brain, fasciculus retroflexus, implying the loss of forebrain control circuitry*. European Neuropsychopharmacology, 2002. **12**: p. 287-297.
- Schmued, L.C. and J.F. Bowyer, *Methamphetamine exposure can produce neuronal degeneration in mouse hippocampal remnants*. Brain Research, 1997. **759**(1): p. 135-140.

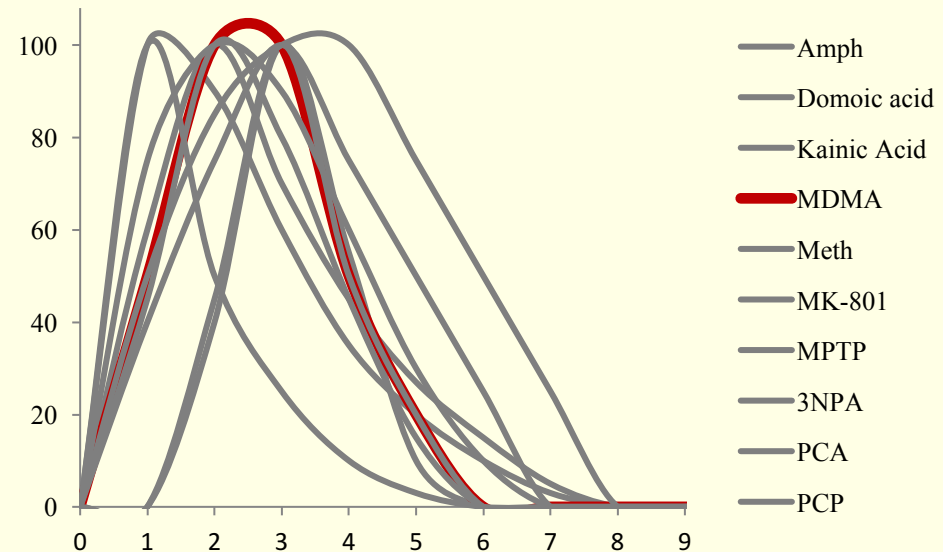
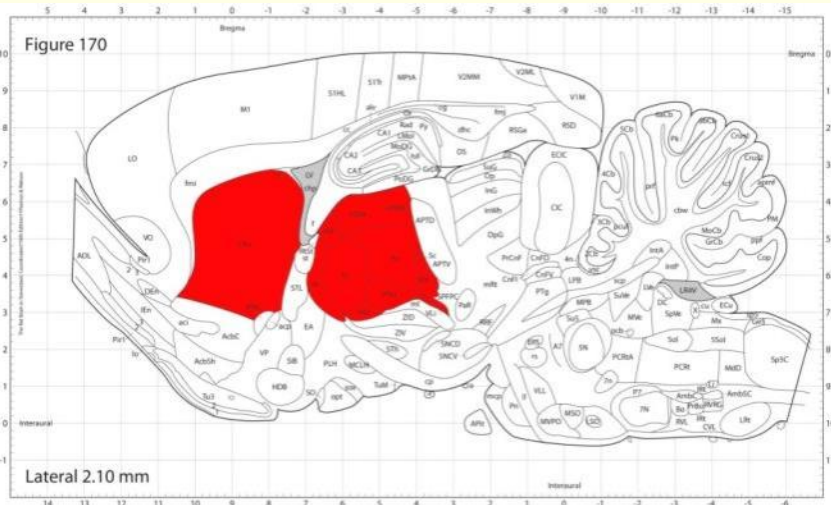
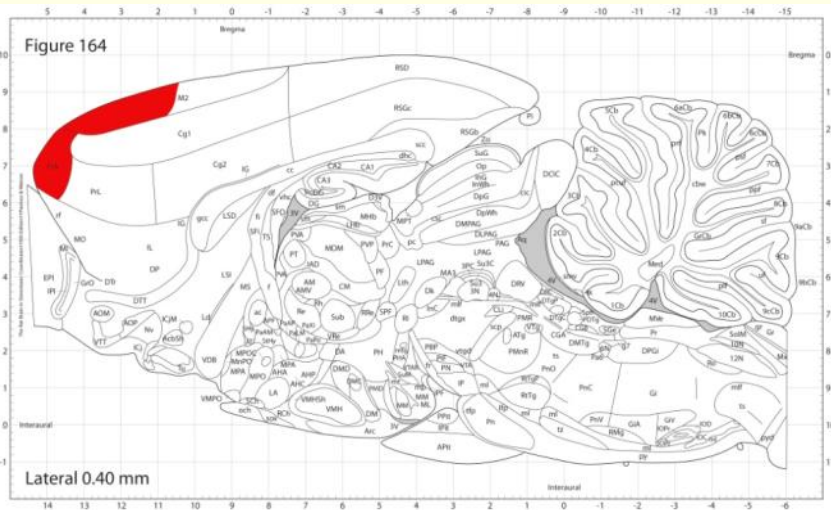
# Acute Neurodegeneration Profile for MDMA

## Location:

- ▶ Degenerating cell bodies can be seen in frontoparietal region of neocortex
- ▶ Degenerating synaptic terminals can be seen in caudate putamen and thalamic nuclei

## Timing

- ▶ 18hrs: Staining percentage was maximal and declined thereafter (representing terminals and axons)
- ▶ 48hrs: degeneration visible in terminals, axons and cell bodies
- ▶ 60hrs: degeneration only slightly reduced from previous
- ▶ 7days: detectable degeneration significantly reduced
- ▶ 14 days post-administration: still detectable degeneration (axons)



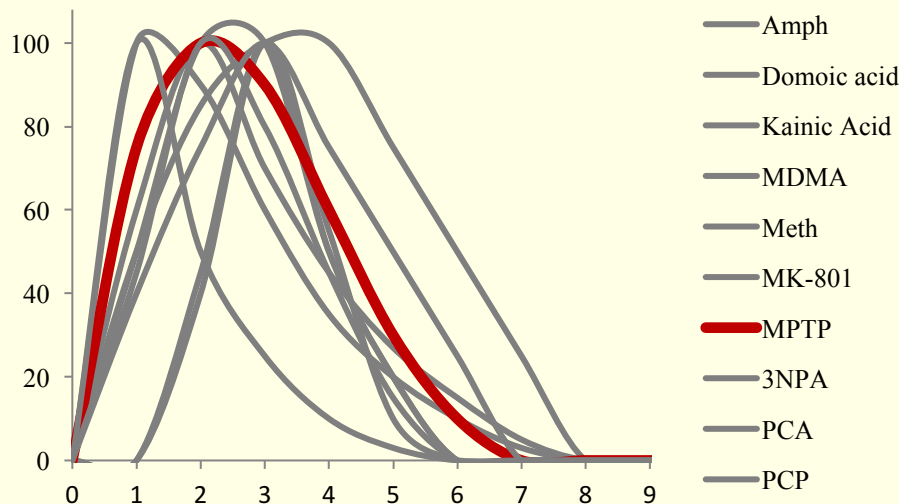
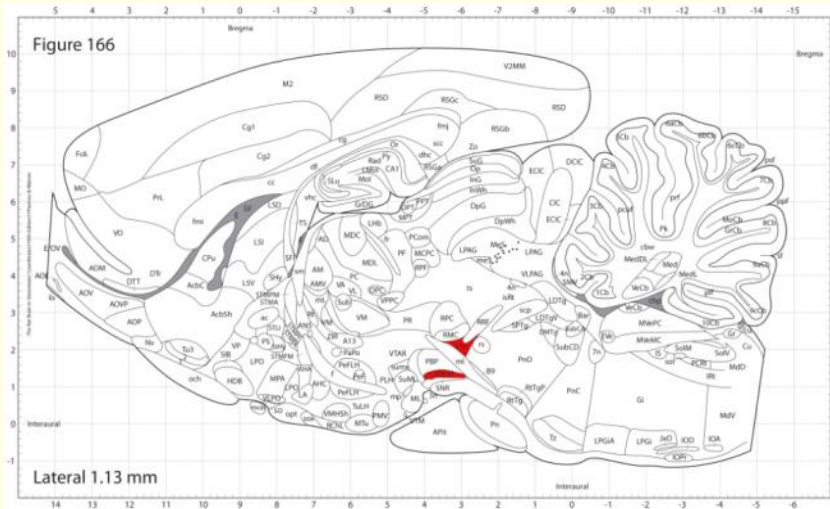
# Acute Neurodegeneration Profile for MDMA

- Carlson, J., et al., *Selective neurotoxic effects of nicotine on axons in fasciculus retroflexus further support evidence that this is a weak link in brain across multiple drugs of abuse*. Neuropharmacology, 2000. **39**(13): p. 2792-2798.
- Ellison, G., *Neural degeneration following chronic stimulant abuse reveals a weak link in brain, fasciculus retroflexus, implying the loss of forebrain control circuitry*. European Neuropsychopharmacology, 2002. **12**: p. 287-297.
- Jensen, K.F., et al., *Mapping toxicant-induced nervous system damage with a cupric silver stain: a quantitative analysis of neural degeneration induced by 3,4-methylenedioxymethamphetamine*, in *Assessing Neurotoxicity of Drugs of Abuse*, L. Erionoff, Editor. 1993, U.S. Department of Health and Human Services: Rockville, MD. p. 133-149.
- Johnson, E.A., J.P. O'Callaghan, and D.B. Miller, *Chronic treatment with supraphysiological levels of corticosterone enhances D-MDMA-induced dopaminergic neurotoxicity in the C57BL/6J female mouse*. Brain Research, 2002. **933**: p. 130-138.
- Johnson, E.A., et al., *d-MDMA during vitamin E deficiency: effects on dopaminergic neurotoxicity and hepatotoxicity*. Brain Research, 2002. **933**: p. 150-163.
- O'Shea, E., et al., *The relationship between the degree of neurodegeneration of rat brain 5-HT nerve terminals and the dose and frequency of administration of MDMA ('ecstasy')*. Neuropharmacology, 1998. **37**: p. 919-926.



# Acute Neurodegeneration Profile for MPTP

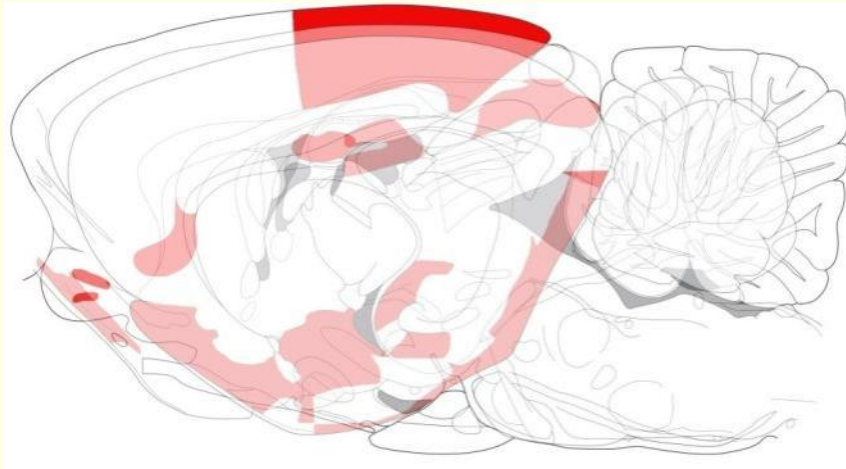
- ▶ Location:
  - ▶ Ventral Tegmental Area
  - ▶ Substantia nigra
- ▶ Timing
  - ▶ 48-60hrs: Peak neurodegeneration staining of nigrostriatal dopaminergic cell bodies, dendrites and axons is observed



Luellen, B.A., et al., *Neuronal and Astroglial Responses to the Serotonin and Norepinephrine Neurotoxin: 1-Methyl-4-(2'-aminophenyl)-1,2,3,6-tetrahydropyridine*. J Pharmacol Exp Ther, 2003. **307**(3): p. 923-931.



# Acute Neurodegeneration Profile for MK801

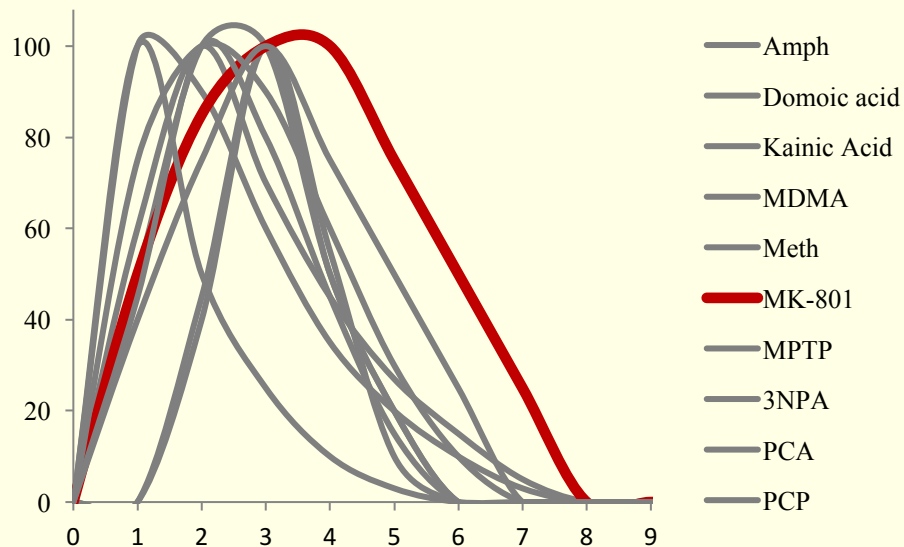


## ► Location:

- retrosplenial cortex;
- dentate gyrus;
- pyriform cortex;
- tenia tecta;
- amygdala;
- entorhinal cortex

## ► Timing

- 1 day post-administration: scattered degeneration, mainly in retrosplenial cortex
- 2 days post-administration: darkly stained neurons observed in all regions listed above
- 3 days post-administration: peak observability of neurodegeneration
- 4 days post-administration: degeneration diminished in many brain regions, but still high in retrosplenial cortex
- 7 days post-administration: degeneration barely detectable



# Acute Neurodegeneration Profile for MK801

- ▶ Ellison, G., *The N-methyl--aspartate antagonists phencyclidine, ketamine and dizocilpine as both behavioral and anatomical models of the dementias*. Brain Research Reviews, 1995. **20**(2): p. 250-267.
- ▶ Fix, A.S., et al., *Neuronal vacuolization and necrosis induced by the noncompetitive N-methyl-D-aspartate (NMDA) antagonist MK(+)-801 (dizocilpine maleate): a light and electron microscope evaluation of the rat retrosplenial cortex*. Experimental Neurology, 1993. **123**(2): p. 204-215.
- ▶ Fix, A.S., et al., *Pathomorphologic effects of N-methyl-D-aspartate antagonists in the rat posterior ingulate/retrosplenial cerebral cortex: A review*. Drug Development Research, 1994. **32**(3): p. 147-152.
- ▶ Fix, A.S., et al., *Integrated Evaluation of Central Nervous System Lesions: Stains for Neurons, Astrocytes, and Microglia Reveal the Spatial and Temporal Features of MK-801-induced Neuronal Necrosis in the Rat Cerebral Cortex*. Toxicologic Pathology, 1996. **24**(3): p. 291-304.
- ▶ Fix, A.S., et al., *Quantitative analysis of factors influencing neuronal necrosis induced by MK-801 in the rat posterior cingulate/retrosplenial cortex*. Brain Research, 1995. **696**: p. 194-204.
- ▶ Olney, J.W., et al., *MK-801 prevents hypobaric-ischemic neuronal degeneration in infant rat brain*. The Journal of Neuroscience, 1989. **9**(5): p. 1701-1704.
- ▶ Olney, J.W., J. Labruyere, and M.T. Price, *Pathological changes induced in cerebrocortical neurons by phencyclidine and related drugs*. Science, 1989. **244**(4910): p. 1360-1362.
- ▶ Olney, J.W., et al., *MK-801 powerfully protects against N-methyl aspartate neurotoxicity*. European Journal of Pharmacology, 1987. **141**: p. 357-361.
- ▶ Olney, J.W., et al., *Environmental agents that have the potential to trigger massive apoptotic neurodegeneration in the developing brain*. Environmental Health Perspectives, 2000. **108**(Supplement 3): p. 383-388.
- ▶ Olney, J.W., *Excitotoxicity, apoptosis and neuropsychiatric disorders*. Current Opinion in Pharmacology, 2003. **3**(1): p. 101-109.
- ▶ Olney, J.W., et al., *Do pediatric drugs cause developing neurons to commit suicide?* TRENDS in Pharmacological Science, 2004. **25**(3): p. 135-139.
- ▶ Wozniak, D.F., et al., *Disseminated corticolimbic neuronal degeneration induced in rat brain by MK801*. Neurobiology of Disease, 1998. **5**(5): p. 305-322.
- ▶ Allen, H.L., et al., *Phencyclidine, Dizocilpine, and Cerebrocortical Neurons*. Science, 1990. **247**(4939): p. 221.
- ▶ Horvath, Z.C., J. Czopf, and G. Buzsaki, *MK-801-induced neuronal damage in rats*. Brain Research, 1997. **753**(2): p. 181-195.
- ▶ Creeley, C.E., et al., *Donepezil markedly potentiates memantine neurotoxicity in the adult rat brain*. Neurobiology of Aging, 2006. **in press**.
- ▶ Creeley, C., et al., *Low Doses of Memantine Disrupt Memory in Adult Rats*. J. Neurosci., 2006. **26**(15): p. 3923-3932.
- ▶ Jevtovic-Todorovic, V., et al., *Early Exposure to Common Anesthetic Agents Causes Widespread Neurodegeneration in the Developing Rat Brain and Persistent Learning Deficits*. J. Neurosci., 2003. **23**(3): p. 876-882.
- ▶ Jevtovic-Todorovic, V., N. Benshoff, and J.W. Olney, *Ketamine potentiates cerebrocortical damage induced by the common anaesthetic agent nitrous oxide in adult rats*. Br J Pharmacol, 2000. **130**(7): p. 1692-1698.
- ▶ Jevtovic-Todorovic, V., et al., *Prolonged exposure to inhalational anesthetic nitrous oxide kills neurons in adult rat brain*. Neuroscience, 2003. **122**(3): p. 609-616.
- ▶ Maas, J.W., Jr., et al., *Calcium-Stimulated Adenylyl Cyclases Modulate Ethanol-Induced Neurodegeneration in the Neonatal Brain*. J. Neurosci., 2005. **25**(9): p. 2376-2385.

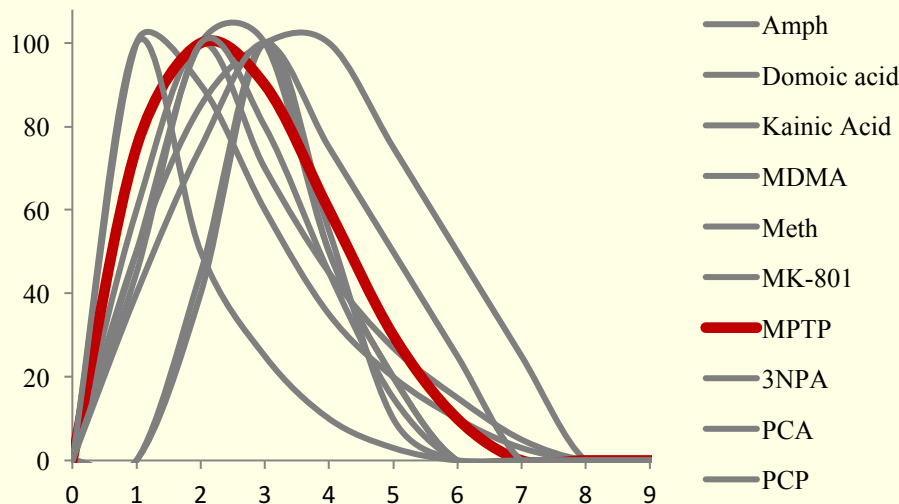
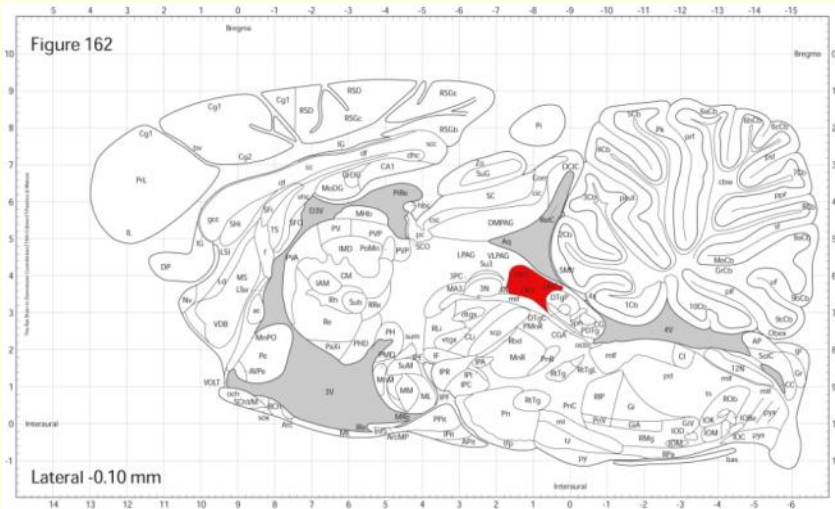
# Acute Neurodegeneration Profile for 2'-NH<sub>2</sub>-MPTP

## ► Location:

- Dorsal raphe

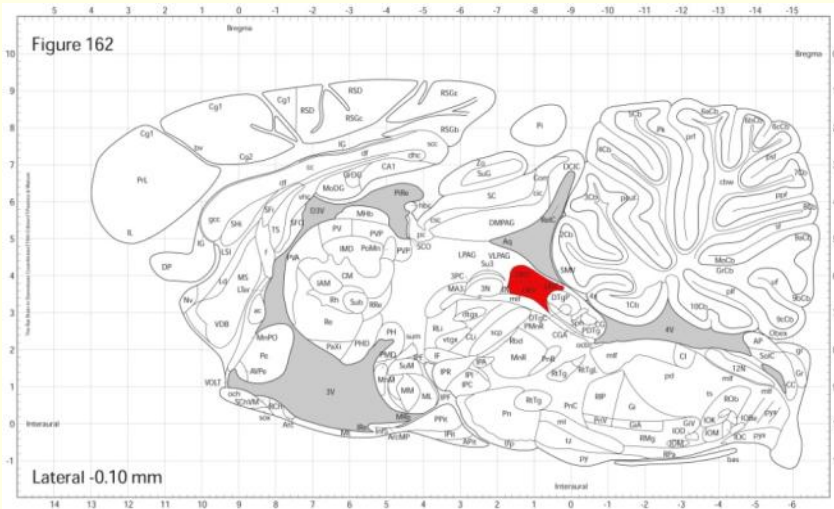
## ► Timing

- 48-60hrs: degenerating cell bodies seen in dorsal raphe; axonal damage seen in median raphe



Luellen, B.A., et al., *Neuronal and Astroglial Responses to the Serotonin and Norepinephrine Neurotoxin: 1-Methyl-4-(2'-aminophenyl)-1,2,3,6-tetrahydropyridine*. J Pharmacol Exp Ther, 2003. **307**(3): p. 923-931.

# Acute Neurodegeneration Profile for PCA p-chloroamphetamine



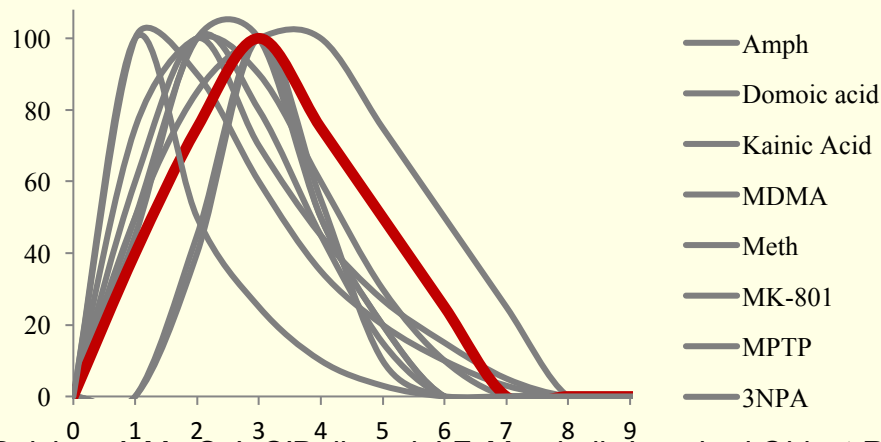
## ► Location:

### ► Serotonergic cells

- Raphe nuclei (B-7 and B-8),
- B-9 serotonergic cell group

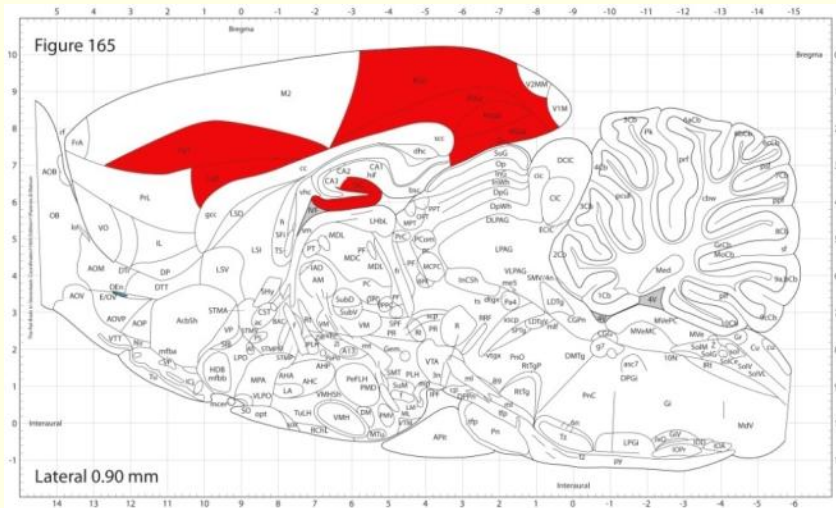
## ► Timing

- 1 day post-administration: degeneration staining observed at all dose levels
- 3 days post-administration: degeneration staining observed at all dose levels
- 9 days post-administration: low intensity of degeneration staining visible after only the highest doses
- 14 days post-administration: low intensity of degeneration staining visible after only the highest doses
- 30 days post-administration: some small continuing degeneration changes witnessed



- Belcher, A.M., S.J. O'Dell, and J.F. Marshall, *Impaired Object Recognition Memory Following Methamphetamine, but not p-Chloroamphetamine- or d-Amphetamine-Induced Neurotoxicity*. *Neuropsychopharmacology*, 2005. **30**(11): p. 2026-2034.
- Wilson, M.A. and M.E. Molliver, *Microglial response to degeneration of serotonergic axon terminals*. *Glia*, 1994. **11**: p. 18-34.
- Harvey, J.A., S.E. McMaster, and L.M. Yunger, *p-Chloroamphetamine: selective neurotoxic action in brain*. *Science*, 1975. **187**(4179): p. 841-843.

# Acute Neurodegeneration Profile for PCP phencyclidine

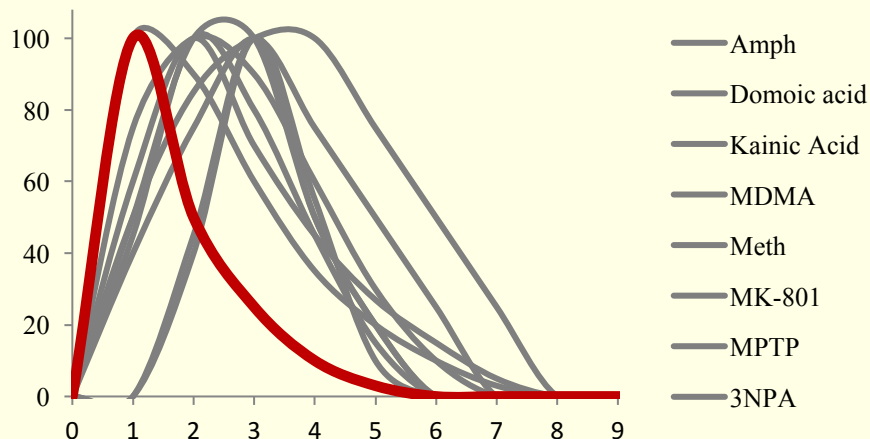


## ► Location:

- Entorhinal cortex
- Dentate gyrus (in ventral hippocampus)
- Cingulate and retrosplenial cortex

## ► Timing

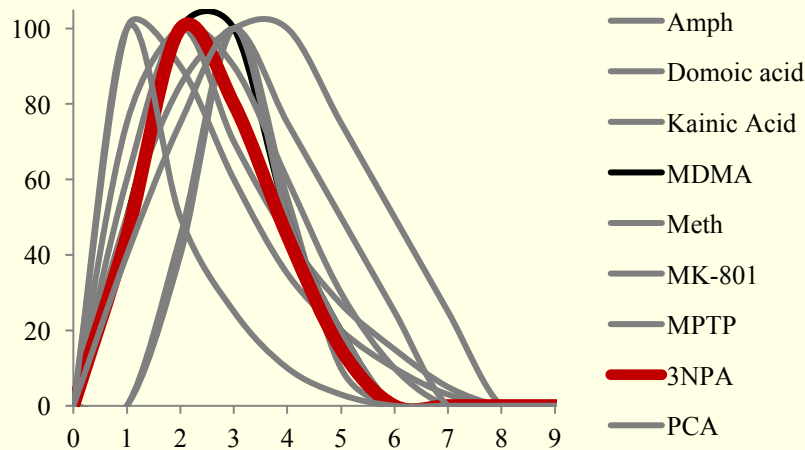
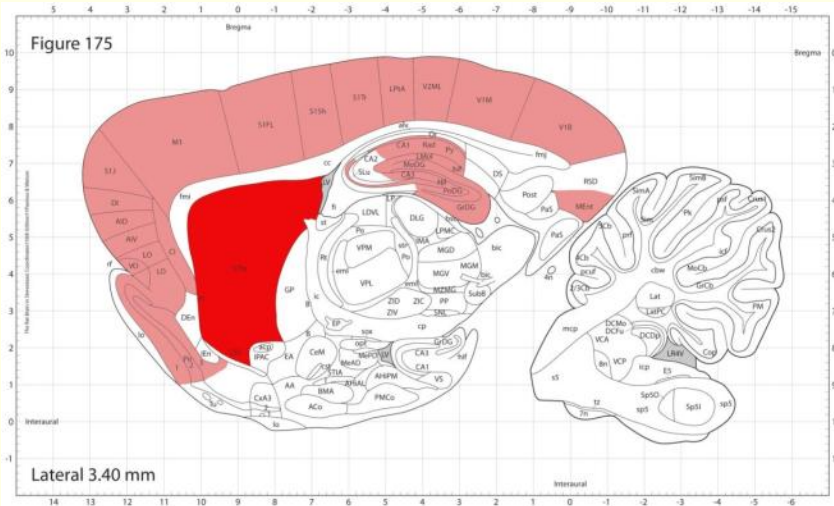
- 24hrs: Peak neurodegeneration staining observed



- Carlson, J., et al., *Selective neurotoxic effects of nicotine on axons in fasciculus retroflexus further support evidence that this a weak link in brain across multiple drugs of abuse*. Neuropharmacology, 2000. **39**(13): p. 2792-2798.
- Ellison, G., *The N-methyl--aspartate antagonists phencyclidine, ketamine and dizocilpine as both behavioral and anatomical models of the dementias*. Brain Research Reviews, 1995. **20**(2): p. 250-267.



# Acute Neurodegeneration Profile for 3-Nitropropionic acid (3NPA)



- ▶ Location:
  - ▶ Caudate putamen
  - ▶ Hippocampus
  - ▶ Many cortical structures (parietal/sensory, temporal/auditory, occipital/visual, frontal/motor, prefrontal, cingulate, piriform, entorhinal, insular)
- ▶ Timing
  - ▶ 2.5 days following administration:
    - widespread dark neuronal staining
    - full Golgi-like staining of the perikaryon and dendritic processes, impregnated axons
    - The presence of uneven staining of the perikaryon and corkscrew-like dendrites indicated an early pathological change
  - ▶ After 15 days: small amounts of neuronal debris present

Miller, P.J. and L. Zaborsky, *3-Nitropropionic acid neurotoxicity: visualization by silver staining and implications for use as an animal model of Huntington's Disease*. Experimental Neurology, 1997. **1146**: p. 212-229.



# Neurotoxins each have a distinct signature

- ▶ Neurotoxins differ in the cell types and locations within the brain that they affect
- ▶ Therefore, the brain needs to be sampled throughout to ascertain neurotoxicity
- ▶ Neurotoxins have slightly differing timing profiles, however there is great overlap for many in their acute toxicity
- ▶ Thus, sampling twice within the first 5 days will often catch any neurotoxicity that exists