The effects of alcohol exposure on the development of the rat superior colliculus
by Tiffany Hardin Selong

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biological Sciences
Montana State University
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Abstract:
Victims of Fetal Alcohol Syndrome (FAS) are known to have visual system dysfunctions; however, with the exception of studies demonstrating damage to the rat optic nerve, experimental studies have not characterized the effects of developmental alcohol exposure on central nervous system (CNS) structures that may be involved. Because the main target of the rat optic nerve is the stratum griseum superficiale (SGS) of the superior colliculus, this study examined the effects of developmental alcohol exposure on cell populations in the SGS using light microscopic stereology.

In the first experiment offspring were exposed to alcohol throughout gestation by maternal consumption of alcohol and were analyzed on postnatal days 15 and 35. In the second experiment artificially reared rat pups were exposed to alcohol via gastrostomy in a high dose "binge" exposure on postnatal days 5 and 6, and were analyzed on postnatal days 15 and 90. In animals exposed throughout gestation alcohol caused a 30% loss of neurons and a 25% reduction in the volume of the SGS at 15 days of age, but there was no longer a loss of neurons at 35 days. However, there was an 83% increase in the volume density of glia and an 85% increase in the total number of glia in the 35 day animals. In the postnatal "binge" exposed animals alcohol caused a 16% reduction in the volume of the SGS and a marginally significant 18% loss of glia (p=0.06) at 15 days. In the same experiment at 90 days of age alcohol caused a 16% reduction in the volume of the SGS and a marginally significant 17% increase in the volume density of neurons (p=0.06). It appears that gestational alcohol exposure delayed neuronal proliferation and migration; this delay was followed by gliosis. Binge postnatal alcohol exposure apparently delayed glial maturation and decreased volume and thus possibly the complexity of the neuropil. These findings correlate with alcohol-induced effects on the developing optic nerve and together with more detailed future studies may help clarify the mechanisms by which alcohol affects developing CNS structures in the visual system.

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Tiffany Hardin Selong

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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STATEMENT OF PERMISSION TO USE

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ABSTRACT

Victims of Fetal Alcohol Syndrome (FAS) are known to have visual system dysfunctions; however, with the exception of studies demonstrating damage to the rat optic nerve, experimental studies have not characterized the effects of developmental alcohol exposure on central nervous system (CNS) structures that may be involved. Because the main target of the rat optic nerve is the stratum griseum superficiale (SGS) of the superior colliculus, this study examined the effects of developmental alcohol exposure on cell populations in the SGS using light microscopic stereology. In the first experiment offspring were exposed to alcohol throughout gestation by maternal consumption of alcohol and were analyzed on postnatal days 15 and 35. In the second experiment artificially reared rat pups were exposed to alcohol via gastrostomy in a high dose "binge" exposure on postnatal days 5 and 6, and were analyzed on postnatal days 15 and 90. In animals exposed throughout gestation alcohol caused a 30% loss of neurons and a 25% reduction in the volume of the SGS at 15 days of age, but there was no longer a loss of neurons at 35 days. However, there was an 83% increase in the volume density of glia and an 85% increase in the total number of glia in the 35 day animals. In the postnatal "binge" exposed animals alcohol caused a 16% reduction in the volume of the SGS and a marginally significant 18% loss of glia (p=0.06) at 15 days. In the same experiment at 90 days of age alcohol caused a 16% reduction in the volume of the SGS and a marginally significant 17% increase in the volume density of neurons (p=0.06). It appears that gestational alcohol exposure delayed neuronal proliferation and migration; this delay was followed by gliosis. Binge postnatal alcohol exposure apparently delayed glial maturation and decreased volume and thus possibly the complexity of the neuropil. These findings correlate with alcohol-induced effects on the developing optic nerve and together with more detailed future studies may help clarify the mechanisms by which alcohol affects developing CNS structures in the visual system.

INTRODUCTION

Fetal alcohol syndrome (FAS) was first classified as a syndrome in 1973 in a classic article by Jones and Smith (1973), in which they described the distinctive pattern of birth defects found in children born to chronic alcoholic women. FAS is characterized by growth deficiency, characteristic facial anomalies, and central nervous system (CNS) dysfunction (Sokol and Clarren,'89; Streissguth et al.,'91). The most serious consequences of FAS are the CNS dysfunctions and anomalies including microencephaly, mental retardation, behavioral deficits, visual and auditory system dysfunction, fine motor deficits, and cerebellar dysfunction (Clarren et al.,'78; Clarren,'81; Streissguth,'86; Driscoll et al.,'90). FAS is the most common cause of mental retardation in the Western world (Abel and Sokol,'87).

Animal research has been essential in characterizing specific CNS effects and the mechanisms of alcohol teratogenicity in the development of the mammalian CNS (Becker et al.,'94). Animal models allow researchers to control such variables as nutritional requirements for pregnant females and their offspring, dosage of alcohol and blood alcohol concentration, and specific timing of alcohol exposures to identify vulnerable periods of CNS development. Many specific regions in the rat CNS have been shown to be vulnerable to developmental alcohol exposure, including the cerebral cortex (Miller,'97), cerebellum (Goodlett et al.,'97), the hippocampal formation (Bellinger et al.,'99), and the olfactory bulb (Bonthius et al.,'92; Maier et al.,'99). The types of alcohol-induced changes that have been demonstrated include neuron cell loss (Bonthius and West,'90), defects in neurogenesis and neuronal migration (Miller,'86; Miller,'95), and astrogliosis (Shetty and Phillips,'92; Goodlett et al.,'97). Most studies have focused on areas of the CNS with well-defined

layers or borders and well-studied developmental histories, while other less well-defined areas have generally been neglected including structures in the visual pathway that are likely affected by alcohol.

Visual anomalies are frequently encountered in human victims of FAS and include optic nerve (ON) hypoplasia, poor vision, and strabismus (Stromland,'90; Stromland et al.,'91; Stromland and Pinazo-Duran,'94; Stromland and Hellstrom,'96). Animal studies investigating the effects of alcohol on the development of the visual system have demonstrated ON hypoplasia and decreased myelination of ON fibers in the mouse (Ashwell and Zhang,'94), reduced myelin thickness and delayed myelination in the rat ON (Phillips,'89; Phillips et al.,'91), loss of ON fibers in the rat (Phillips et al.,'98), delayed migration and maturation of neurons in the rat oculomotor nucleus (Burrows et al.,'95), and altered maturation of neurons in both the kitten lateral geniculate nucleus (Magloczky et al.,'90) and in the rat and mouse superior colliculus (SC) (Zajac,'87; Wall and Phillips,'93; Ashwell and Zhang,'94). It is known that for proper connections to be made during CNS development, including the development of the visual system, adequate input stimuli must exist (Galli-Resta et al.,'93). If alcohol-induced changes alter visual system input, developing target structures of the CNS might also be affected (Simon et al.,'94). Such effects on target structures in the visual system could contribute to some of the neurological problems frequently associated with FAS such as poor vision, strabismus, poor eye-hand coordination, and mental retardation (Jones and Smith,'75; Streissguth et al.,'91; Rosenberg,'96).

The SC, an integrative nucleus in the visual pathway, is one of the main targets of the ON fibers from the retina in the rat (Sefton and Dreher,'95). Because developmental alcohol exposures have been shown to produce significant changes in the rat ON in terms of both nerve fiber numbers and myelination (Phillips,'89; Phillips et al.,'91; Phillips et al.,'96; Phillips et al.,'98), it is reasonable to hypothesize that significant effects will be seen in the SC, particularly since activity-dependent input has been shown to be essential to the normal development of the SC or optic tectum of several

species, including rats (O'Leary et al.,'86; Williams et al.,'94; Cramer and Sur,'96; Rajan and Cline,'98).

The specific goals of this project were to complete analyses of previously collected SC tissues taken from rats exposed to alcohol in two different experimental models. One model is a gestational exposure in which pregnant dams were fed alcohol throughout gestation and is the equivalent of alcohol exposure during the first two trimesters of human gestation (Wall and Phillips,'93). The other model is a 5-6 day (d) postnatal binge exposure which used artificially reared postnatal rats fed dietary alcohol via gastrostomy and is the equivalent of acute alcohol exposure during the third trimester of human gestation (Phillips et al.,'98). Light microscopic stereology and immunocytochemistry were used to analyze the effects of these alcohol exposures on the neuronal populations that receive retinal input and their associated glial cell populations in the superficial (visual) layers of the SC.

The light microscopic methods utilized in this study were based on principles of modern stereology. Stereology, as applied to quantitative neurohistology, is a collection of methods that estimate the volume density of cells in a structure, the total number of cells in a structure, and the volume of defined regions of the brain. Stereological methods use three dimensional (3-D) probes called "disectors" to deal with cells as 3-D structures. These methods are considered "unbiased" because assumptions do not have to be made about cell size, shape, or orientation, and are called "design-based" because the precision with which one can estimate the true value of cell density or total number of cells in a defined region is related to the actual stereological sampling design one employs to obtain the estimate of cell density, total volume, and total cell number.

LITERATURE REVIEW

Fetal Alcohol Syndrome

Fetal alcohol syndrome (FAS), first described as a syndrome in 1973, is characterized by a distinctive pattern of birth defects found in children born to chronic alcoholic women (Jones and Smith,'73). Characteristics of FAS commonly include growth deficiency, characteristic facial anomalies, and central nervous system (CNS) dysfunction (Sokol and Clarren,'89; Streissguth et al.,'91). The most serious consequence of FAS is the CNS dysfunction (physically characterized by microencephaly) that is manifested as lifelong disabilities including mental retardation, hyperactivity, poor fine-motor function, and auditory and visual dysfunction (Clarren,'81). In 1987, FAS was reported as the most common cause of mental retardation in the Western world, creating a substantial economic impact on our society (Abel and Sokol,'87; Abel and Sokol,'91) Results of longitudinal clinical studies indicate that the intellectual, behavioral, and neurological handicaps attributed to FAS do not improve over time, even with good care given by parents, doctors, and educators (Streissguth et al.,'91; Stromland and Hellstrom,'96).

Clinical studies have shown that the development of the human visual system is affected by alcohol exposure in utero (Aronson et al.,'85; Stromland,'90; Stromland and Hellstrom,'96). The ocular and visual anomalies frequently associated with FAS include optic nerve hypoplasia, poor vision, ptosis, strabismus, and tortuous retinal arteries (Holzman et al.,'90; Stromland,'90; Chan et al.,'91; Stromland et al.,'91; Stromland and Pinazo-Duran,'94; Stromland and Hellstrom,'96). FAS has also been correlated with severe delays in development of visual perception and with poor eye-hand coordination (Aronson et al.,'85; Aronson et al.,'97). Thus, the insult of developmental alcohol

exposure on the human visual system reaches from the physical development of the eyes to the development of visual perception. Since "visual perception" is a higher order CNS function, this suggests that alcohol affects some part of the visual pathway, from the eye to higher levels in the CNS. "Perception" is usually thought of as a function of the cerebral cortex, but a complex behavior such as eye-hand coordination certainly involves additional CNS structures in the visual pathway such as the superior colliculus, which in the human helps coordinate head and eye movement with visual stimuli (Nolte,'99).

For proper connections to be made during CNS development, including development of the visual system, adequate input stimuli must exist (Williams et al.,'94; Cramer and Sur,'96; Rajan and Cline,'98). If visual input is altered by ocular or optic nerve (ON) manipulations or deformities, the developing target structures of the CNS are also affected (Galli-Resta et al.,'93; Simon et al.,'94). Such effects on target structures may contribute to neurological problems such as poor vision, mental retardation, and poor eye-hand coordination (Jones and Smith,'75; Streissguth et al.,'91; Rosenberg,'96).

Studies in human victims of FAS are limited mainly to clinical characterizations that, except for magnetic resonance imaging (MRI) (Mattson et al.,'94, Roebuck et al.,'98) and autopsy (Clarren et al.,'78; Peiffer et al.,'79; Wisniewski et al.,'83; Clarren,'86), rarely include tissue examination. MRI studies have shown microencephaly with substantial reduction in volume of particular structures in the CNS including the cerebellum, basal ganglia, diencephalon, and corpus callosum (Mattson et al.,'94). Autopsy studies have shown fairly consistent alcohol-induced anomalies in CNS development including microencephaly, malformations of the corpus callosum and cerebellum, and glial heterotopias (Clarren et al.,'78; Peiffer et al.,'79; Wisniewski et al.,'83; Clarren,'86). Variables that are impossible to control in human studies such as nutrition, multiple drug use, and environmental influence, often confound results (Becker et al.,'94). Thus, various animal models

have been implemented to investigate how alcohol affects the development of the brain and other

organs. The experimental design of such models controls for nutrition and environment, the

manipulation of dosage, and the timing of exposure to pinpoint specific developmental events.

Nutrition is an important variable since it is possible for nutritional deficits alone to cause some of

the same effects seen in FAS (Weinberg,'84; Wiggins,'86; Phillips,'92) and because FAS children are

borne of alcoholic women who are often malnourished.

Animal Models: Methods of Developmental Alcohol Exposure

Animal models of developmental alcohol exposure, and particularly the rat animal model,

have evolved and become quite sophisticated in recent years allowing for proper nutritional controls

(Lieber and Decarli,'94), and allowing specific developmental events to be targeted (West,'93). The

rat model using both gestational and early postnatal exposures has been used extensively in this

laboratory to investigate the effects of alcohol on brain development (Phillips,'89; Phillips et al.,'91;

Shetty and Phillips,'92; Phillips,'94; Burrows et al.,'95; Phillips et al.,'97). In terms of relative brain

development, the full three trimesters of human gestation is equivalent to the 21-22 day gestation of

a rat plus the first 10 postnatal days (Dobbing and Sands,'79; Dobbing,'81). Therefore, a rat

gestational exposure is approximately equivalent to an exposure occurring during the first two

trimesters of human gestation, and exposure during the first 10 postnatal days is approximately

equivalent to an exposure occurring during the third trimester of human gestation.

Several different methods have been used to expose fetal rats to alcohol during gestation,

including exposing the mother via intubation, injection, and either dietary or drinking water inclusion

(Weinberg,'84). Though dose can be strictly controlled by both intubation and injection, these

methods and the intoxication from the alcohol are stressful to the dams causing them to decrease diet

intake and introduce concerns about adequate nutrition (Weinberg,'84). Alcohol inclusion in

drinking water provided as the only source of fluid causes experimental dams to decrease both fluid and diet intake, leading to malnutrition (Testar et al.,'88). Including alcohol in liquid diet provided as the only source of fluid and diet is generally acknowledged as the best method for gestational alcohol exposure (Rao et al.,'88; Lieber and Decarli,'94). The liquid diets are formulated to minimize any effects of decreased food intake because of alcohol content (Lieber and Decarli,'94). The pair-fed method of the present study involves feeding pregnant dams liquid diet as the only source of water and food, and feeding a calorically equivalent equal volume to weight-matched control dams. This ensures that nutrition is controlled for and that the dose can be monitored. It has been shown that the offspring of pair-fed animals do not differ significantly from the offspring of chow-fed controls in terms of litter size, offspring size, and brain weight of offspring, or in terms of developmental markers such as righting reflex, dental eruption, startle response, and eye opening (Gottesfeld et al.,'89).

Several different methods have been used to expose neonatal rat pups to alcohol, including lactational exposure, gavage, inhalation, injection, and artificial rearing (Swiatek et al.,'86; Phillips,'94). Lactational exposure presents the risk of malnutrition due to the altered nursing behavior of the pups and decreased milk production by lactating dams (Swiatek et al.,'86). Gavage, inhalation, and injection also present the risk of altered nursing behavior in the intoxicated rat pups. The postnatal rat exposure used in the present study involves an artificial rearing technique, similar to the method developed in the lab of James West (West et al.,'84; West,'93). This technique controls for nutrition, while leaving the dose and timing of alcohol administration available for manipulation (West, J.R., Goodlett, C.R., and Kelly, S.J. (1987)). To my knowledge the present study is the first to use a postnatal artificial rearing technique to examine the effects of alcohol exposure on the development of the rat superior colliculus.

The Superior Colliculus

The superior colliculus of the rat midbrain is equivalent to the optic tectum of submammalian vertebrates such as the chick and the frog, and is an important relay nucleus of the rat visual pathway (Sefton and Dreher,'95). Most of the retinal ganglion cells (RGCs), whose axons comprise the optic nerve (ON), project to the SC in the rat while others (fewer in number) project to the lateral geniculate nucleus of the thalamus (LGN) (Linden and Perry,'83; Sefton and Dreher,'95). In the human, the main target of the ON is the LGN with a smaller projection directly to the SC. Thus the human SC is a lesser part of the direct visual system but remains a relay point in the pathway for coordinating head and eye movement responses to visual input (Nolte,'99).

The rat SC is an integrative nucleus that projects to the substantia nigra, pons, medulla, cervical spinal cord, and other visual nuclei such as the oculomotor nucleus (Sefton and Dreher,'95; Nolte,'99). Similarly, the human SC projects to the cervical spinal cord, mesencephalic reticular formation, and the lateral and medial geniculate nuclei (Nolte,'99). The SC coordinates eye and neck movements in response to visual and other sensory stimuli (Sefton and Dreher,'95) and thus functions in helping an organism respond to where an object is in space. It has also been implicated in a variety of other visual activities including coordinating avoidance reactions and saccadic eye movements (Sefton and Dreher,'95).

Organization of the Superior Colliculus

The adult rat SC appears in histologic section as a well-laminated structure with seven horizontal layers of alternating grey and white matter. The laminae can be divided into two functional regions, the superficial (visual) laminae and the deep (projection) laminae. The visual laminae are the focus of the present study and from superficial to deep consist of: stratum zonale (SZ); stratum griseum superficiale (SGS); and stratum opticum (SO) (Figure 1). The SO consists
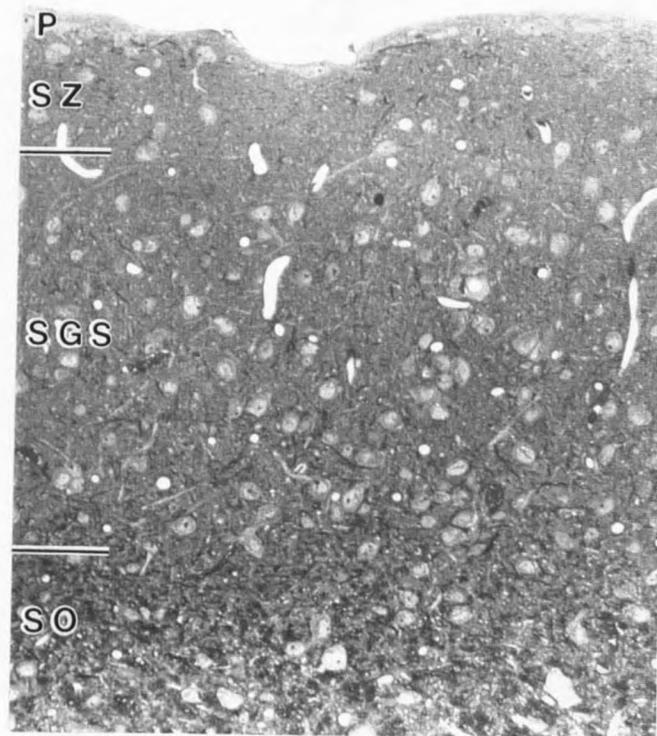
Figure 1. Light micrograph of a 1 μm coronal section through the superficial (visual) laminae of the left superior colliculus of a 90 day old rat, stained with toluidine blue. The pial surface (P) is at the top. Note: the transversely sectioned myelinated fibers in the stratum opticum (SO); the high density of cells in the stratum griseum superficiale (SGS); and the relative lack of cells in the stratum zonale (SZ). Magnification is 175X.

mainly of rostrocaudally oriented myelinated nerve fibers from the ON and afferents from other parts of the visual system such as the visual cortex. The nerve fibers from the ON enter the nucleus in the SO, then after a longitudinal course, turn superficially to synapse in the SZ and outer SGS on neurons whose cell bodies are found in the SGS (Lund,'72). The SZ is a molecular layer consisting mainly of neuronal processes and synapses. Neurons in the SGS project axons to the deep laminae where visual information is integrated with input from other sources, such as from the somatosensory cortex (sensory modalities) and the inferior colliculus (auditory input) (Sefton and Dreher,'95).

Golgi techniques have been used to classify six neuron types in the superficial laminae of the mature rat SC, all of which are assumed to receive input from the ON (Langer and Lund,'74; Labriola and Laemle,'77; Warton and Jones,'85), and include marginal cells, horizontal cells, piriform cells, stellate cells, narrow field vertical cells, and wide field vertical cells. The piriform cells, narrow field vertical cells, and wide field vertical cells are projection neurons that are primarily found in the SGS and that project axons to the deep integrative laminae of the SC. Piriform cells are located in the outer SGS and have cup-shaped somata with dendrites projecting superficially and axons originating from the base. Narrow field vertical cells are located in the SGS and have fusiform shaped somata oriented perpendicular to the pial surface. Wide field vertical cells are located in the deep SGS near the border of the SO and have shapes similar to that of the more superficial piriform cells. The horizontal cells, marginal cells, and stellate cells are fewer in number and are considered local interneurons in the superficial SC. Horizontal cells are located along the zone between SZ and outer SGS and their processes are arranged tangential to the pial surface. Marginal cells are located in the SZ and their processes project deeper within the superficial laminae of the SC. Stellate cells are randomly oriented multipolar neurons found throughout the superficial layers but which are most numerous in the SO and the SGS.

Development of the Rat Superior Colliculus

Neurogenesis, Histogenesis, and Synaptogenesis

Neurogenesis in the rat SC has been studied using autoradiographic techniques (Labriola and Laemle,'77; Altman and Bayer,'81). In these studies, animals were given an intraperitoneal or amniotic sac injection of systemic tritiated thymidine (thymidine labeled with ^{3}H) that was incorporated into DNA during replication (Angevine and Sidman,'61). Initially, cells incorporating ^{3}H-thymidine were heavily labeled, but after pulse labeling the signal became more dilute as the cells continued to divide. Experiments of this type can identify cells that were "born" during the period of time ^{3}H-thymidine was available for incorporation into DNA. By tracing when cells were born and to where these cells migrate, it has been possible to demonstrate the time course of origin of specific cells in the SC and their migration routes to form definitive layers (Labriola and Laemle,'77; Altman and Bayer,'81).

As the neuroepithelial cells that form the wall of the neural tube become layered, several zones can be distinguished (Boulder Committee,'70). The ventricular zone surrounds the lumen of the neural tube and eventually becomes the ependymal lining of the ventricles of the brain and central canal of the spinal cord. The ventricular zone is initially characterized by a high mitotic rate. As the wall of the tube thickens, a subventricular zone can be distinguished and becomes the main site of cell proliferation. Cells migrate out of the proliferative zones and eventually differentiate into the neurons and glia of the CNS. The intermediate zone extends from the subventricular zone to the marginal zone and the marginal zone extends to the pial surface (Boulder Committee,'70). The intermediate zone is composed of postmitotic migrating neurons and the marginal zone is formed by their processes. Neuronal cell bodies from the intermediate zone organize into the gray matter of the CNS in the spinal cord and brain stem, and their myelinated axons become the white matter.

Neurons are thought to migrate along radial glia from the ventricular and subventricular zones to their respective locations in cerebral cortex (Rakic,'81). In the chick diencephalon early migration does occur radially, but tangential migration of neurons along axonal tracks accounts for a significant part of later neuronal migration (Golden et al.,'97). Similarly, radial migration is followed by tangential migration of neurons in the chick brain stem (Hemond and Glover,'93). Studies in the rat SC have verified that its development is similar to other parts of the CNS, where neurons are derived from the ventricular and subventricular zones of the embryonic mesencephalic tectum and are thought to be guided during neuronal migration by the processes of radial glia (Mustari et al.,'79; Ganzler-Odenthal and Redies,'98), but to my knowledge tangential migration has not been studied in the rat brain stem.

Neurogenesis in the rat SC takes place on gestational days (G) 12 - 18 with both a rostrocaudal and an inside-out gradient of development (Bruckner et al.,'76; Mustari et al.,'79; Altman and Bayer,'81; Raedler et al.,'81). Thus, the oldest neurons are located rostral in the SC and in the deepest layer. One group suggests a more complicated gradient where the deep layers have an inside-out sequence of development but the superficial layers have a complex outside-in gradient of development (Altman and Bayer,'81). Neurogenesis specific to the superficial laminae occurs in a more restricted temporal window than that of the entire SC, from G15 - 18 (Mustari et al.,'79; Altman and Bayer,'81) (see Figure 16 in the Discussion).

The postnatal development of neurons in the superficial layers of the SC has been studied by Golgi-Cox impregnation methods (Labriola and Laemle,'77; Warton and Jones,'85). At 3d, neurons have immature morphology with few branched dendrites that develop further through elongation and branching. Adult neuron types can be identified by 9-15d. Dendritic spines can be seen as early as 9d, but most develop between 23-31d. The larger neurons, such as the vertical-type neurons, develop

earlier than the smaller neurons, such as the marginal and stellate cells (Labriola and Laemle,'77; Warton and Jones,'85).

Neuron development in the superficial SC can also be characterized by the expression of specific neurotransmitters. In the cat SC γ-aminobutyric acid (GABA), an inhibitory neurotransmitter, is expressed early in neuronal development during neuronal migration (Mize et al.,'96). GABA is found mainly in the superficial SC and even though immunoreactivity can be seen early on, it seems that GABAergic inhibition develops postnatally. Glutamate, which is involved in most retinotectal synapses, is expressed during synaptogenesis and fiber refinement which occur from birth through 31d (Warton and McCart,'89; Sakurai and Okada,'92).

Nervous system development generally involves an initial overproduction of neurons and subsequent elimination by apoptosis or programmed (or naturally occurring) cell death (Cowan et al.,'84). Cell death during the normal development of the rat SC occurs from G20 to 11d with the peak rate of cell death on the day of birth (Arees and Astrom,'77), which is coincident with neuronal migration and differentiation in the rat SC (Labriola and Laemle,'77; Warton and Jones,'85). Cell death is thought to occur normally for two reasons: (1) to match the size of the afferent RGC population with the size of the target SC neuronal population, and (2) to eliminate neurons that are connected improperly in the formation of the retinotopic map (Bunt et al.,'83; Jeffery,'84). Developing RGCs also go through a period of naturally occurring cell death that occurs over the first 10 postnatal days in the rat (Cunningham et al.,'82), during which the cell population drops from ~200,000 to ~117,000 and ON fibers drop from ~250,000 to ~120,000 (close to adult numbers) (Perry et al.,'83). The period of cell death in the superficial layers of the SC and the ganglion cell layer of the retina overlap, and the days at which neuronal cell death appears maximal in both populations coincide at 6-7d (Cunningham et al.,'82). Afferent input is necessary to mediate naturally occurring cell death in the SC (Cunningham et al.,'79; Galli-Resta et al.,'93). If the

spontaneous firing of the retina is silenced on 6d by the application of tetrodotoxin (TTX), cell death in the rat SC is increased (Galli-Resta et al.,'93). A similar increase in cell death occurs in the rat SC if enucleation is performed at birth (Giordano and Cunningham,'82). At least two possibilities exist to explain these effects on cell death: (1) electrical stimulation of ON fibers releases a trophic factor that sustains neurons in the SC, or (2) activity of the ON fibers is directly trophic to the target neurons in the SC. These studies indicate that cell death is a normal part of SC development and that the process of cell death is modified by afferent input from the ON. The SC has also been implicated in modulating RGC cell death (Huxlin et al.,'95). The SC has been shown to produce proteoglycan which was hypothesized to act as a trophic factor for RGCs, and when this proteoglycan was injected into the early postnatal rat retina, it rescued a significant number of RGCs from apoptosis (Huxlin et al.,'95).

The development of the retinocollicular pathway results in a precise retinotopic map (Lund,'72; Diao et al.,'83; Simon and O'Leary,'91) in which greater than 90% of the afferent axons from each retina project to the contralateral SC (Linden and Perry,'83). Rat optic tract axons enter the SC in the SO and run in a rostrocaudal direction, and then turn superficially to synapse in the superficial laminae of the SC (Langer and Lund,'74). These axons have en passant synapses in the zone of vertical cells in the SGS but the greatest density of terminal synapses occurs in the SZ. Most retinal axons synapse with the dendrites of wide and narrow field vertical cells of the SGS although it is assumed that all neurons in the SGS receive ON input (Warton and Jones,'85; Boyes and Veronesi,'88). Nasal retina projects to the caudal SC, temporal retina projects to the rostral SC, dorsal retina projects to the lateral SC, and ventral retina projects to the medial SC (Diao et al.,'83). To achieve this precise pattern, normally developing RGC axons must grow out from the retina to form the optic nerves; decussate at the optic chiasm to form the optic tracts; select a target (primarily SC in the rat); form initial diffuse synaptic contacts within the SC; and refine synaptic connections to

form a precise retinotopic map on the SC. The current paradigm classifies mechanisms involved in the development of the retinocollicular projection as either activity-independent or activity-dependent (Goodman and Shatz,'93). The activity-independent mechanisms are predominantly responsible for early development of the retinocollicular projection including neurite outgrowth and axonal guidance and are based on molecular guidance cues and trophic factors (Goodman and Shatz,'93). Activity-dependent mechanisms are predominantly responsible for later development of the retinocollicular projection including synaptogenesis and refinement of the retinotopic map. Activity-dependent mechanisms include both spontaneous afferent electrical activity from the retina up until eye opening at around 12-14d (Lund and Lund,'72; Galli-Resta et al.,'93), and patterned visual activity from the retina after eye opening (Lund and Lund,'72; Simon et al.,'94).

Activity-independent processes are guided by the molecular interactions of cell adhesion molecules in both cell-cell and cell-substrate interactions, and by diffusible chemical gradients (Hankin and Lund,'91; Goodman and Shatz,'93). In the rat, target extract from the developing SC has been shown to stimulate neurite outgrowth from RGCs in vitro (Bosco et al.,'93). Nerve growth factor (NGF) has also been shown to stimulate the outgrowth of neurites but in a nonspecific manner, causing growth of processes from many types of retinal cells (Bosco et al.,'93). Since SC extract has been shown to be type-specific for RGCs, it appears that RGC development is differentially regulated by soluble signals from the target tissue. In the mouse, evidence supports the hypothesis that pioneer RGC axons lead the way out of the retina forming a pathway for the next axons to follow in the formation of the ONs (Silver and Sidman,'80). RGC axons must choose whether to cross or not to cross at the optic chiasm, where more than 90% decussate to innervate the contralateral SC (Linden and Perry,'83). From the optic chiasm the nerve fibers form the optic tracts and must find the appropriate target in the SC. The first afferents from the retina arrive at the rat SC on G16 (Bunt et al.,'83).

Retinal afferents are prevented from crossing the tectal midline by glial cells that function as part of the substrate that helps compartmentalize axons during retinal afferent innervation (Wu et al.,'98). On G13 the median ventricular formation (MVF) appears in the dorsal mesencephalic wall (Raedler et al.,'81). As the MVF develops, it appears to contain many parallel fibers of astrocytes projecting towards the pial surface and likely contributes to the formation of the midline glial septum. In hamsters, it has been found that the midline glial septum plays a role in compartmentalizing the optic input to the SC (Wu et al.,'98). If the glial processes of the midline septum are lesioned, axons can be induced to aberrantly cross the midline indicating that these glia have a barrier function. The midline glial septum in the rat also functions to compartmentalize the retinal input to the SC (Snow et al.,'90).

The development of a precise retinotopic map from an initially diffuse retinal projection to the SC depends on competitive interactions between axons with the correct topographic target and axons projecting to an aberrant location (Simon et al.,'94). The ipsilateral retinocollicular projection is larger in the neonate than in the adult (Laemle and Labriola,'82). This projection increases in density from birth to 6d but then gradually recedes until the adult projection is attained by about 10d (Perry et al.,'83). Many of the axons that initially reach the SC have collaterals that branch to connect to a wider area of the SC. When contralateral innervation is removed by enucleation in the newborn rat, the ipsilateral projection does not recede and instead forms functional synapses (Laemle and Labriola,'82). The period of synaptic proliferation in the SGS extends through 30d and is followed by a period of synaptic elimination until the adult synaptic density is attained by about 85d (Warton and McCart,'89). Neural activity accounts for the strengthening of some synapses and the elimination of others. There is a 'critical period' during which normal vision (activity) is necessary for development of the precise retinocollicular map that occurs from eye opening to 30-35d (Lund and Lund,'72).

Activity-dependent mechanisms account for the refinement of neural connections, including the refinement of the retinotopic map in the SC and are thought to involve glutamate activation of N-methyl-D-aspartate (NMDA) receptors on the postsynaptic neurons (Shatz,'90; Chen and Tonegawa,'97). One hypothesis is that NMDA receptor activation on the postsynaptic neuron causes an influx of Ca^{2+}, which is required for calmodulin to stimulate the enzymatic synthesis of nitric oxide (NO) by nitric oxide synthase (NOS) (Garthwaite,'91). NO can then diffuse across the cell membrane, and is thought to be a retrograde messenger which can stimulate guanylate cyclase in the presynaptic cell and raise intracellular levels of the second messenger, cyclic guanosine monophosphate (cGMP) (Garthwaite,'91). Although the specific role of cGMP is still unclear, it has been shown to act directly on ionic channels of neuronal cell membranes, neurotransmitter production and release, and growth cone motility (Garthwaite,'91), and through these actions may be involved in the refinement of the retinotopic map.

Several lines of evidence implicate the NMDA-NO mechanism in the activity-dependent development of the retinocollicular (or retinotectal, depending on the species) projection. In the development of the chick retinotectal projection, which at maturity is completely contralateral, a transient ipsilateral projection is present which persists if NOS in the tectum is inhibited (Wu et al.,'94). Since NO is normally expressed in chick tectal cells and inhibiting its synthesis affects the refinement of the retinotopic map, NO is likely a messenger from tectal cells for feedback to the retinal cells during development (Wu et al.,'94). The transient chick ipsilateral retinotectal projection will also persist if the NMDA receptor itself is blocked, which directly implicates NMDA receptor activation in the refinement of the retinotopic map (Ernst et al.,'99). This particular activity-dependent mechanism is not universal for connections involving other neurotransmitters such as acetylcholine, as the cholinergic fiber patches that develop in the rat SC are not affected by inhibiting NOS (Mize et al.,'97). Further, NOS expression by tectal cells in the chick optic tectum is dependent

upon the presence of ON fibers (Williams et al.,'94). Thus, if the eyes are removed during early

development, NOS expression significantly decreases, which further implicates NO as a retrograde

messenger. The specific role of NO in refinement is not understood, but there is evidence that NO

stops axonal elongation when an appropriate target is contacted by causing the collapse of RGC

growth cones, and it is suggested to be an important messenger in stabilizing synapses (Gally et

al.,'90; Hofer and Constantine-Paton,'94).

NOS, the enzyme that produces NO, is expressed during the time period corresponding to

the refinement of synapses (Mize et al.,'96). In the chick optic tectum, NOS expression has been

correlated with the period of optic nerve innervation but then peaks before hatching during the initial

period of synaptogenesis (Williams et al.,'94). There appears to be a different temporal expression

of NOS-positive staining in the rat (Tenorio et al.,'95). NOS-positive cells are first seen on 7d, with

peak expression on 15d. Thus, NOS expression correlates with RGC cell and axon death and

synaptic refinement (Perry et al.,'83).

Since there is good evidence that NMDA receptor activation and NO feedback are involved

in the development of the retinocollicular projection, studies examining the effects of alcohol on the

development of neurons expressing NOS in the SC have been started in this laboratory (Phillips et

al.,'99). Gestational alcohol exposure decreased the areal cell density of NOS-containing neurons in

the SGS indicating either a cell loss or a loss of NOS expression. The implication of these findings

is that gestational alcohol exposure could alter the developing connectivity of the neurons in the SGS

and, therefore, could have further ramifications on cell death and neuronal maturation.

Gliogenesis

Macroglia (astrocytes and oligodendrocytes) of the SC are distributed in a pattern and

express molecules similar to elsewhere in the CNS (Harvey et al.,'93). During CNS development

astrocytes form limiting membranes (Snow et al.,'90), induce and maintain the blood-brain barrier formed by the endothelial cells of CNS blood vessels (Janzer and Raff,'87; Janzer,'93), secrete growth factors (Martin,'92), play a role in axonal guidance (Silver and Sidman,'80), enhance neuronal survival (O'Malley et al.,'94), and stimulate neuronal morphogenesis (Chamak et al.,'87). In the mature CNS, astrocytes play such roles as enhancing the efficacy of synapses (Pfrieger and Barres,'97), transporting substances between blood and neurons (Abbott,'87), and cycling glutamate (Rothstein et al.,'96). Oligodendrocytes are the myelinating cells of the CNS (Compston et al.,'97) but can also play an inhibitory role in axon growth (Schwab and Caroni,'88). Microglia are macrophage-derived immunocompetent cells in the CNS (Davis et al.,'94; Compston et al.,'97). These cells migrate into CNS tissue from the periphery during embryogenesis and are thought to form the mature resident population (Davis et al.,'94). Microglia perform phagocytic functions to remove cellular debris resulting from natural cell death during development and in response to injury and disease (Davis et al.,'94). Microglia were not specifically analyzed in the present study.

There are few references in the literature that directly investigate ghogenesis in the rat SC although inferences can be made from experiments examining glial development in other regions of the CNS. Tissue transplant studies in rats have shown that macroglial precursor cells are present by G15 in the SC (Harvey et al.,'93). In a qualitative study of the development of the rat visual system, gliogenesis in the SC was found to be similar to that of the visual cortex (Raedler and Sievers,'75) and, therefore, studies of glial development in the rat visual cortex can provide clues to similar events in the SC. Autoradiography of the postnatal formation of macroglia in the rat visual cortex showed that gliogenesis occurs in two peaks (Mares and Bruckner,'78). The first peak from 3-7d is due primarily to astrocyte proliferation and occurs during the period of growth and maturation of neurons. The second peak on 16d is due primarily to oligodendrocyte proliferation and occurs at the onset of myelination (Mares and Bruckner,'78). This same pattern of macroglial proliferation, in

which astrocytes are generated before oligodendrocytes, should occur in the development of the SC. A postnatal assay for two enzymes used as specific glial markers in the rat CNS [glutamine synthetase - astrocytes; 2',3' cyclic nucleotide phosphohydrolase (CNPase) - oligodendrocytes and myelin], suggests large differences in the functional maturation of these two glial cell populations: metabolic activity increases only modestly in astrocytes after birth; CNPase has a steep increase between 8-14d in oligodendrocytes (Virgili et al.,'90). This supports the idea that astrocytes are already present and functional in the rat CNS at birth, whereas oligodendrocytes generally mature postnatally. In the hamster, oligodendrocytes mature and myelination occurs along the proximo-distal axis of the ON fibers in the visual pathway, such that even myelination of the SO occurs in a rostrocaudal direction (Jhaveri et al.,'92). Therefore, the timing of myelination for different components of the rat visual system is expected to be different depending upon where the structure lies physically along the CNS visual pathway. Myelination of the SO begins on 12d (Warton and Jones,'85) and occurs relatively late in comparison to myelination of the ON, which begins on 6d (Tennekoon et al.,'77). In the present study the development of myelin was not studied; however, immunocytochemical staining was used to characterize the effects of alcohol on the development of the astrocytes in the SC.

Antibodies to glial fibrillary acidic protein (GFAP) and S-100 were used as markers to study astrocytes (Schmidt-Kastner and Szymas,'90). GFAP is an intermediate filament located in the cytoplasm of astrocytes (Schmidt-Kastner and Szymas,'90). It is important to recognize that in the study of GFAP+ cells, GFAP expression in astrocytes is being characterized rather than a direct study of astrocyte numbers or process size (Hawrylak and Greenough,'95). S-100 is a calcium-binding protein that is specific to astrocyte soma and cell processes in the CNS (Ghandour et al.,'81; Wijsman and Shivers,'93). In the normal mature SC, heavy GFAP staining is localized to the external limiting membrane, to the area surrounding blood vessels, and to the midline of the tectum,

but more diffuse staining is found throughout the SGS (Harvey et al.,'93). Anti-GFAP has been used to stain CNS tissues from rats developmentally exposed to alcohol, and alcohol has been shown to cause transient astrogliosis in the cerebrum and cerebellum as judged by the increased number of reactive astrocytes which stain GFAP+ (Shetty and Phillips,'92; Goodlett et al.,'93).

Previous Work with Alcohol on the Development of the Visual System

Humans with FAS have ocular anomalies and visual dysfunctions (Stromland,'90; Stromland et al.,'91; Stromland and Pinazo-Duran,'94; Stromland and Hellstrom,'96). Animal studies have helped clarify which structures in the mammalian visual pathway are vulnerable to developmental alcohol exposure, and to some extent have helped to define the temporal windows of vulnerability of these structures. Damage to the retina and the eye caused by developmental alcohol exposure would obviously affect normal vision. Electroretinography was used to examine the functioning of photoreceptors in dark-adapted rats after developmental alcohol exposure and showed increased threshold and latency with decreased response amplitude (Katz and Fox,'91). Developmental alcohol exposure in rats has been shown to cause the improper migration of RGCs and 25% reduction in the thickness of the neural retina during early development (G16-4d) (Ferriero et al.,'92), and in the mouse has been shown to cause micropthalmia (Cook et al.,'87), and increased cell cycle time and cell loss during ocular ontogeny (Kennedy and Elliot,'86). Alcohol-induced cell loss and/or changes in the electrophysiology of photoreceptors in the retina may alter the activity of the RGCs, and so could help explain any changes in the activity-dependent developmental processes that occur in the development of the SC

The development of the alcohol-exposed ON has been examined extensively using animal models of FAS in this and other laboratories. Both a full three trimester human equivalent alcohol exposure and a third trimester human equivalent alcohol exposure caused a delay in myelin

acquisition, a permanent reduction in the myelin thickness (Phillips,'89; Phillips et al.,'91), and a delay in oligodendrocyte maturation (Phillips and Krueger,'90; Phillips and Krueger,'92). Postnatal "binge-like" alcohol exposures given at either 5-6d or 9-10d both caused significant developmental alterations at 15d, including reductions in the cross-sectional area of the ON and thinner myelin on ON axons (Phillips et al.,'96). Particularly relevant to the present study, the 5-6d binge exposure caused a significant reduction (-40%) in the number of nerve fibers in the ON at both 15 and 90d (Phillips et al.,'98). In the present study, SC tissues from the same 5-6d binge-exposed animals were analyzed at both 15d and 90d. Using a gestational alcohol exposure, Stromland and Pinazo-Duran ('94) reported a significant decrease in both ON glial cell density and the number of ON fibers, and delayed myelination in rat ON on 4d and 7d. In a very different exposure model whereby mice were exposed to ethanol in utero via intraperitoneal injection on G8, 15d ON was found to have a significantly reduced cross sectional area, a reduced number of ON axons, and a reduced percentage of myelinated axons (Ashwell and Zhang,'94). Because alcohol caused significant developmental changes in the ON in several alcohol exposure experiments, it may also alter the development of the connectivity of the neurons in the superficial laminae of the SC.

The rat oculomotor nucleus innervates extraocular eye muscles and receives some input from the SC, and is also vulnerable to developmental alcohol exposure (Burrows et al.,'95). After gestational alcohol exposure, there was a reduction in the cross-sectional area of the oculomotor nucleus, a decrease in the density of neurons, and an increase in the number of astrocytes in the nucleus. Neurons normally clustered in the center of the nucleus were more diffusely arranged at 15d, indicating that alcohol can either delay maturation of or permanently alter the structure of the oculomotor nucleus.

Studies of other regions of the visual pathway after alcohol exposures are limited. Gestational alcohol exposure has been shown to cause significant delay in the maturation of neurons

in the kitten LGN, as indicated by the extent of dendritic arbor in Golgi-Cox stained neurons (Magloczky et al.,'90). To my knowledge there have been no studies in the visual cortex in animal models of FAS.

Though the normal development of the SC has been well-studied in the rat, little research has focused on its development during or after alcohol exposure. G18 mice exposed to ethanol in utero were reported to have an increase in the neuronal areal density in the superficial SC, accompanied by a reduced total area of the midbrain, and a reduction in the ratio of midbrain cross sectional area to that of the cerebral aqueduct (Zajac et al.,'88). This study is hard to interpret because only G18 mice were analyzed and although the experimental dams were fed a commercially obtained alcohol-inclusive liquid diet, a pair-fed paradigm was not used so it is likely that the control group was not a proper control for nutrition. Ashwell and Zhang ('94) looked at the mouse 15d ON and SGS after an acute exposure to ethanol by intraperitoneal injection on G8. While they found significant differences in the cross-sectional areas of ON, a reduction in the number of ON axons, and deficient myelination, no significant differences were reported in the total size of the neuronal population in the superficial SC. However, alcohol caused an increase in the areal density of SGS neurons and a decrease in the total volume of the SC, which led the authors to conclude that the estimated total number of neurons was unaltered. This experiment suggests alcohol-induced changes to the neuronal population of the SC in the absence of changes in absolute neuronal number, even under the limited exposure to ethanol the mice received.

Studies in this lab have provided some evidence of significant alcohol-induced effects on the development of the superficial lamina of the rat SC (Wall and Phillips,'93; Phillips et al.,'99). In a pilot study, pregnant dams ingested alcohol throughout gestation via liquid diet, and male offspring were examined at 15 and 35d (Wall and Phillips,'93). Tissues were processed for either plastic embedding or Golgi impregnation. Alcohol caused a decrease in the complexity of the dendritic

arbors of one class of Golgi-stained neurons in the SGS (Wall and Phillips,'93). Light microscopic

analysis of toluidine blue-stained plastic sections at 15d found no statistically significant differences

in either neuronal or glial areal densities. That experiment was repeated in part using more

consistent tissue sampling to produce the tissues of the present study. One set of SC tissues from the

present exposure was stained for nitric oxide (NO) producing cells by the use of histochemical

staining for nitric oxide synthase (NOS) (Phillips et al.,'99). Analysis at 15d found that alcohol

caused a significant reduction (-24%) in the number of NOS+ neurons in the SGS of the SC (Phillips

et al.,'99). NO is a neural messenger molecule that has been implicated in many roles in the CNS

including as a messenger transiently expressed in the tectum during the development of synaptic

connections (Wu et al.,'94). Since NOS+ neurons appear to be important for the acquisition of

proper circuitry during SC development and NOS+ neurons are affected in the SC by gestational

alcohol exposure, it is important to further characterize the development of the SC in alcohol-

exposed animals.

Principles of Stereology

Neural Counting Methods

There are a variety of neural counting methods that have been used to quantify either the

areal or volume density, or total number of cells in defined regions of the nervous system. Such

methods include serial reconstruction, profile counts, assumption-based methods, and stereology (3-

D) (Oorschot,'94; Coggeshall and Lekan,'96). Each of these methods has appropriate uses and

distinct advantages and disadvantages. Currently, the issue of which methods are most appropriate

for analysis of individual CNS regions is hotly debated in the literature (Geinisman et al.,'97; Hagg et

al.,'97; Peterson et al.,'97; Schmitz,'97).

Counts derived from serial section reconstruction of an entire defined region or nucleus determine the actual number of cells within a defined region (Coggeshall and Lekan,'96). This is the most absolute neural counting method available. Briefly, the region of interest is exhaustively sectioned and the histological sections are analyzed in sequence to reconstruct the entire structure. Profiles of cells are followed from section to section such that each cell is counted once. The advantage to this method is that the number of cells counted is the true number, not an estimation that may be biased. The disadvantage is that this method is inefficient and extremely labor intensive, and can be limited by technical inconsistencies such as missing sections.

Less accurate but most commonly used methods of cell counting are 2-D profile counts whereby profiles of cells are sampled and counted on histologic sections to estimate areal densities or total numbers of cells (Oorschot,'94; Coggeshall and Lekan,'96; Morrison et al.,'98). The assumption is that changes in the areal density or number of profiles are representative of changes in the true volume density or total number of cells. Intuitively, one can imagine that larger cells will create more profiles than smaller cells. Another way to view this concept is that the profile of a larger cell has a higher probability of being on any section than does the profile of a smaller cell. Therefore, one disadvantage of these methods is that if profiles of different size cells are being counted, then total profile numbers may not be equal to total cell numbers. In addition, if an experimental manipulation changes cell volume, then comparing profile areal densities or total profile numbers between experimental and control groups would lead to difficulties in interpretation. These methods are considered biased because profiles of larger cells have a higher probability of being counted. The advantage of these methods is that they are relatively quick and simple. If it can be shown that cell volume is not affected by experimental manipulation, then relative changes in profile number/density are probably similar to real changes in cell number/density. However, since

the true number/density of cells is not estimated, interpretations of changes resulting from profile

counts are still open to criticism.

Cell counting methods designed to correct for the overestimation of cell numbers in simple

profile counts are the model-based methods that convert profile numbers to cell numbers using

correction factors (Abercrombie,'46; Coggeshall and Lekan,'96). These methods have also been

called assumption-based because they make geometric assumptions about cells in order to create the

correction factors. For instance, the assumption is that all nuclei are spherical. A correction factor

based on this assumption is entirely dependent on how well the cell nuclei fit the assumption. The

most commonly used model-based method is the method of Abercrombie (Abercrombie,'46). The

advantages to these methods are that they are efficient and accurate as long as the assumptions are

met. The disadvantage is that there is no way to know if the estimations are biased unless the chosen

method is calibrated for the cell type and region of interest using serial section reconstruction.

Stereological cell counting methods utilize sampling designs based on modified serial section

reconstructions and are considered more accurate than simple profile counts and model-based counts

(Sterio,'84; Gundersen,'86; Coggeshall and Lekan,'96; Mayhew and Gundersen,'96). These methods

make no assumptions about size, shape, or orientation of cells, and are therefore considered

unbiased. The assumptions which must be met are that every object has an equal probability of

being counted and that profiles of cells can be followed from one section to the next. The simplest

form of modern stereology is the physical disector consisting of two parallel sections of tissue

separated by some distance defining a volume. All cells are counted which appear in the first section

(reference section) but not in the second section (look-up section) (Sterio,'84). The distance between

the two sections can be no greater than the height of the smallest object being counted; this insures

each cell has an equal probability of being counted. Profiles of cells are analyzed on both sections,

and cells are counted when a profile appears on one section but is not found in the next. Because

cells are counted in three dimensions, the physical disector yields an estimate of cell density (cells per unit volume, Nv). The volume of the brain region or structure (reference volume, Vref) can be determined by Cavalieri's method of volume estimation (Gundersen and Jensen,'87). In Cavalieri's method of volume estimation, a systematic (equally spaced along one axis of the structure) sample of sections through the structure is used to measure the area of the structure on each section. The sum of the areas is multiplied by the section separation to yield an estimation of volume, the reference volume. The cell density estimate can then be multiplied by the reference volume, to obtain an estimate of the total number of cells (N) in a structure. The advantage of stereological methods is that they yield unbiased estimates of cell counts and are efficient compared to serial reconstructions. The disadvantages are that they require more work than simple profile counts or assumption-based methods, and can be difficult to apply to structures with poorly defined margins.

As described above, the cell counting method has unique advantages and disadvantages. When determining which method to employ, there are certainly many factors to consider. The estimates of neuron number as determined by profile counts, the method of Abercrombie, and the physical disector have been compared to a serial section reconstruction of 965 neurons in a DRG (Coggeshall et al.,'94). The profile counting method overestimated the true number of neurons counted in the serial section reconstruction by 204% (1979±68 neurons) . The Abercrombie method underestimated the total number of neurons by 46% (527±86 neurons). Only the physical disector provided a precise estimation of cell numbers (969±136 neurons).

The Importance of Calculating N

Many proponents of stereology strongly suggest that the definitive data to be compared should be absolute number of cells, or N, rather than cell density, or Nv (cells per unit volume) (Oorschot,'94). An example of why this is so is apparent in a study in which developmental alcohol