Conduction Block and Glial Injury Induced in Developing Central White Matter by Glycine, GABA, Noradrenalin, or Nicotine, Studied in Isolated Neonatal Rat Optic Nerve

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KEY WORDS
axon; glia; development

ABSTRACT
The damaging effects of excessive glutamate receptor activation have been highlighted recently during injury in developing central white matter. We have examined the effects of acute exposure to four other neurotransmitters that have known actions on white matter. Eighty minutes of Glycine or GABA-A receptor activation produced a significant fall in the compound action potential recorded from isolated post-natal day 10 rat optic nerve. This effect was largely reversed upon washout. Nicotinic acetylcholine receptor (nAChR) or adrenoreceptor activation with noradrenalin resulted in an ~35% block of the action potential that did not reverse during a 30-min washout period. While the effect of nAChR activation was blocked by a nAChR antagonist, the effect of noradrenalin was not ablated by α- or β-adrenoreceptor blockers applied alone or in combination. In the absence of noradrenalin, co-perfusion with α- and β-adrenoreceptor blockers resulted in nonreversible nerve failure indicating that tonic adrenoreceptor activation is required for nerve viability, while overactivation of these receptors is also damaging. Nerves exposed to nAChR + adrenoreceptor activation showed no axon pathology but had extensive glial injury revealed by ultrastructural analysis. Oligodendroglia exhibited regions of membrane vacuolization while profound changes were evident in astrocytes and included the presence of swollen and expanded mitochondria, vacuolization, cell processes disintegration, and membrane breakdown. Blinded assessment revealed higher levels of astrocyte injury than oligodendroglial injury. The findings show that overactivation of neurotransmitter receptors other than those for glutamate can produce extensive injury to developing white matter, a phenomenon that may be clinically significant.

INTRODUCTION
Synaptic signaling between neurons is mediated by the vesicular release of neurotransmitters, an event that can also occur between neurons and glia. Functional glutamatergic synapses onto NG-2(+) glial cells have been identified in various brain regions in immature and mature animals (Paukert and Bergles, 2006), while vesicular glutamate signaling between axons and neighbouring NG-2(+) cells has been documented in the corpus callosum and immature rat optic nerve (Kukley et al., 2007; Ziskin et al., 2007). Ionotropic glutamatergic receptors are expressed by both astrocytes (e.g., Gallo and Ghiani, 2000; Verkhratsky and Kirchhoff, 2007) and oligodendroglia (e.g., Fern and Moller, 2000; Follett et al., 2000; Gallo and Ghiani, 2000; Karadottir et al., 2005; McDonald et al., 1998; Salter and Fern, 2005), where there appears to be a polarized expression with NMDA receptors largely found on the processes and non-NMDA receptors on the somata (Salter and Fern, 2005). The presence of glutamate receptors on oligodendrocytes confers sensitivity to excitotoxic injury, which may be significant clinically, in particular for conditions associated with developing white matter injury such as cerebral palsy (Fern and Moller, 2000; Follett et al., 2000; Salter and Fern, 2005; Wilke et al., 2004).

A number of neurotransmitters other than glutamate have been shown to have actions upon central white matter, in particular during neonatal development. Several studies report endogenous GABA in the neonatal optic nerve (Lake, 1992; Ochi et al., 1993; Sakatani et al., 1992), while GABA-A receptor activation produces a partial nerve block in this preparation (Sakatani et al., 1991, 1992). This effect is associated with extracellular [K+] accumulation and axon depolarization (Sakatani et al., 1994; Simmonds, 1983). Neonatal optic nerve nicotinic acetylcholine receptor (nAChR) activation has a similar effect upon action potential conduction (Zhang et al., 2004), and the actions of both GABA-A and nAChR activation are largely reversible. β-Adrenoreceptor or serotonin receptor activation also reversibly effects axon excitability in this preparation (Honmou and Young, 1995; Saruhashi et al., 1997), while glycine receptor activation has a powerful depolarizing effect upon the
membrane potential of neonatal optic nerve axons (Simmonds, 1983). The significance of the expression of neurotransmitter receptors other than those for glutamate in developing central white matter for injury has not been examined and previous studies have concentrated upon brief periods of receptor activation. In the current study, we test the injury capacity of a standard 80-min period of glycine, nACh, adrenoreceptor, or GABA receptor activation upon neonatal rat optic nerve. nAChR or adrenoreceptor activation were found to produce nonreversible injury characterized by partial conduction block and glial cell pathology. The results reveal the potential for nonglutamate-mediated neurotransmitter-mediated injury in developing matter injury.

**METHODS**

**Ethical Approval**

All procedures were approved by local ethical review and conformed to UK home office regulations. Animals were killed by vertebral dislocation.

**General**

Optic nerves were excised from Lister-hooded rats aged between postnatal days 7 and 15 (referred to as “P10” throughout), placed in an interface perfusion chamber (Medical Systems, Greenvale, NY), and incubated for 60 min before recordings were initiated. The nerves were maintained at 37°C and perfused at a rate of 1.5–2.0 mL/min with artificial cerebrospinal fluid (aCSF). The composition of the aCSF was as follows (in mM): NaCl, 126; KCl, 3; NaH₂PO₄, 2; MgSO₄, 2; CaCl₂, 2; NaHCO₃, 26; glucose, 10; pH, 7.45, bubbled with 5% CO₂/95% O₂. The osmolarity was measured and adjusted to 310–320 mOsm by addition of NaCl, as required. Isolated nerves were continually aerated with 95%O₂/5%CO₂ at a flow rate of 3 L/min.

**Electrophysiology**

Extracellular compound action potentials were evoked and recorded with suction electrodes. Individual nerves were electrically stimulated using square-wave constant current pulses of 150–300 µs duration (Iso stim A320, WPI), and compound action potentials were recorded via a second electrode at the other nerve end (Cyber Amp 320, Axon Instruments). The recorded signal was subtracted from a parallel differential electrode, filtered (low pass: 800–10,000 Hz), digitized (25,000 Hz: 1401 mini, Cambridge Electronic Design), and displayed on a PC running Signal software (Cambridge Electronic Design) with positive (relative to the subtraction electrode) going up. Peak-to-peak amplitude was used to assess changes in the number of unitary action potentials in the neonate, since action potential area cannot be reliably measured in recordings from neonatal nerves. This is due to the large stimulus artefact produced by the long stimulus duration that is required to elicit a full neonatal compound action potential (Fern et al., 1998; Foster et al., 1982).

All reagents used were purchased from Sigma-Aldrich (UK), except for picrotoxin and propranolol hydrochloride, which were purchased from Tocris (UK). The noradrenalin hydrochloride solutions included 0.6 mM ascorbic acid to reduce oxidation. Experiments where noradrenalin or its antagonists were used were carried out in a dark room. The concentrations of neurotransmitters and antagonists used in this study were chosen based upon published reports that have previously studied effects in isolated neonatal rat optic nerve (see “Discussion”).

**Electron Microscopy**

Control nerves were perfused for 120 min with aCSF and were compared with nerves perfused for 80 min with 100 µM noradrenalin + 50 µM nicotine followed by 30-min washout using the standard protocol. Following perfusion, nerves were washed in Sorenson’s buffer prior to postfixation in 3% glutaraldehyde/Sorenson’s for 90 min at room temperature. They were then washed in Sorenson’s 3 × 5 min and stored at 5°C. Nerves were subsequently treated with 2% osmium tetroxide and dehydrated in ethanol and propylene oxide. Sections were counterstained with uranyl acetate and lead citrate and examined with a Jeol 100CX electron microscope; see Thomas et al. (2004) for further details. The preparation of the ultrathin sections for analysis was performed blind by technical staff not associated with the project.

Digital images of individual glial cells were processed using Photoshop software (Adobe, San Jose, CA) and randomly allocated for blinded analysis of cell injury (performed by SC). The scoring system used was as follows: 0 points = no significant pathology in the cell; 1 point = some small vacuoles present in the cytoplasm; 2 points = large vacuoles present in the cytoplasm and/or the presence of swollen mitochondria; 3 points = frank necrosis with breakdown of the cell membrane. Cells were identified as either astrocyte or oligodendroglia using well-established criteria for this preparation. In brief, oligodendroglial cells had a narrow endoplasmic reticulum, numerous mitochondria often clustered at one pole of the cell, a clear cytoplasm containing a large nucleus with regular, dispersed chromatin. Astrocytes featured a wide-bore endoplasmic reticulum, dark bodies in the cytoplasm, an irregular nucleus with chromatic clumped under the envelope and had a darker cytoplasm; see Thomas et al. (2004) and Vaughn (1969) for further details.

**Statistics**

Results are presented as mean ± SEM. Significance was determined by analysis of variance (ANOVA) using Tukey’s post-test or by t-test, as appropriate.
As previously reported (Fern et al., 1998), compound action potential recordings from P10 rat optic nerve were stable during control perfusion with aCSF (action potential amplitude was 103.3 ± 5.9% of the initial value after 120 min of perfusion with aCSF; n = 5; Fig. 1B,C). Perfusion with aCSF containing 100 μM of the inhibitory neurotransmitter glycine produced a gradual fall in compound action potential amplitude to 66.9 ± 7.1% at the end of the period of glycine perfusion (P < 0.005 vs. control; n = 6), recovering to 80.4 ± 7.7% upon washout (not statistically different from control, P > 0.05). This effect was prevented by co-application of the selective glycine antagonist strychnine (2 μM; Fig. 1B).

The cholinergic agonist nicotine (50 μM) evoked a gradual decline in the P10 optic nerve compound action potential (Fig. 2A,C). Action potential amplitude fell to 74.3 ± 8.3% after 80-min exposure (P < 0.005 vs. control; n = 6).

**RESULTS**

As previously reported (Fern et al., 1998), compound action potential recordings from P10 rat optic nerve were stable during control perfusion with aCSF (action potential amplitude was 103.3 ± 5.9% of the initial value after 120 min of perfusion with aCSF; n = 5; Fig. 1B,C). Perfusion with aCSF containing 100 μM of the inhibitory neurotransmitter glycine produced a gradual fall in compound action potential amplitude to 66.9 ± 7.1% at the end of the period of glycine perfusion (P < 0.005 vs. control; n = 6), recovering to 80.4 ± 7.7% upon washout (not statistically different from control, P > 0.05). This effect was prevented by co-application of the selective glycine antagonist strychnine (2 μM; Fig. 1B).

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Perfusion with 100 μM of the adrenoreceptor agonist noradrenaline also resulted in a gradual decline in compound action potential amplitude, reaching 63.6 ± 6.7% of the initial value after 80 min (n = 6, P < 0.001 vs. control; Fig. 3A,B). As with nicotine, the fall in action potential amplitude evoked by noradrenaline was not reversed during a 30-min washout period (amplitude was 70.2 ± 7.1% of the initial value after washout; P < 0.005 vs. control). The effect of noradrenaline was not significantly reduced by the β-adrenoceptor blocker propranolol (10 μM added 10 min prior to exposure to noradrenaline and washed out with the neurotransmitter; Fig. 3B). Co-perfusion of propranolol and the α-adrenoceptor antagonist phentolamine (10 μM added as before), only served to increase the rate of action potential decline produced by noradrenaline (Fig. 3B). This curious result may be explained by the direct actions of adrenoreceptor blockers upon the compound action potential. Perfusion with 10 μM propranolol alone produced a fall in action potential amplitude to 69.1 ± 6.9% after 80-min exposure (n = 7; P < 0.001 vs. control; Fig. 3C). Washing out the β-adrenoceptor antagonist resulted in action potential recovery to 86.0 ± 11.0% (P > 0.05 vs. control). The decline in the action potential evoked by propranolol was not enhanced by co-perfusion with phentolamine, while exposure to phentolamine alone had no significant effect (Fig. 3C). However, co-application of propranolol + phentolamine resulted in incomplete recovery during washout (73.1 ± 8.9%; P < 0.01 vs. control). Block of tonic adrenoreceptor activation therefore resulted in a degree of nonreversible block of optic nerve conduction, while overactivation of adrenoreceptor had a similar effect. This suggests a complex relationship between adrenoreceptor activation and nerve viability, with either too much or too little adrenoreceptor activation leading to nonreversible effects. The reversible component of the effect of propranolol + phentolamine presumably explains the degree of recovery seen when these antagonists are washed off following co-application with adrenalin (Fig. 3B).

Nicotine or noradrenaline applied alone produced a largely nonreversible conduction block in the P10 optic nerve and the effects of co-application of both neurotransmitter were examined. Examples of compound action potentials recorded before, at the end of the 80-min exposure period and after 30 min of washout are shown in Fig. 4A for 50 μM nicotine, 100 μM noradrenaline, and 50 μM nicotine + 100 μM noradrenaline. The effect of co-exposure upon compound action potential amplitude is shown in Fig. 4B, revealing a 64.0 ± 4.8% decline (n = 11, P < 0.001 vs. control), with no significant recovery. The extent of conduction block and recovery for all three conditions is summarized in Fig. 4C, revealing no significant difference between them. This may indicate a common mode of action between these conditions.

To examine the nature of the nonreversible effects of co-application of 50 μM nicotine + 100 μM noradrenaline, nerves were fixed at the end of the 30-min recovery period and processed for ultrastructural analysis. Control nerves perfused with aCSF for 120 min contained axons, astrocytes, and oligodendroglia of normal appearance for this stage in development (Fig. 5A,B). The cell types were distinguished using well-established criteria (see “Methods”), and all cellular compartments were largely free from pathological changes. The health of glial cells was assessed blind using a simple scoring system (see “Methods”), revealing low levels of injury in the control perfused nerves and greatly increased levels of injury in the treated nerves (Fig. 5C). The level of injury was sig-
significantly greater in astrocytes compared with oligoden-
droglia in the treated nerves ($P < 0.005$). Typical oligo-
dendroglia from treated nerves are shown in Fig. 6, and
exhibited an unusual localized bubbling or vesiculation
of the somata cell membrane with retention of relatively
normal cytoplasmic inclusions such as mitochondria and
endoplasmic reticulum. The unusual vesicular break-
down of the cell membrane could also be observed in
oligodendrocyte processes engaged in ensheathing and
myelinating axons (e.g., Fig. 6B: "***"). Axon cylinders

Fig. 4. Combined effects of nicotine and noradrenalin. A: Compound action potentials recorded before (top), during (middle), and after (bottom) 80 min of perfusion with either 50 µM nicotine, 100 µM nor-
adrenalin, or 50 µM nicotine + 100 µM noradrenalin. B: Data plot showing the nonreversible decline in compound action potential amplitude produced by an 80-min exposure to both neurotransmitters. C: Mean decline in the compound action potential recorded at the end of the 80-min exposure and at the end of the recovery period for perfusion with either 50 µM nicotine, 100 µM noradrenalin, or 50 µM nicotine + 100 µM noradrenalin. Error bars are SEM, scale bars = 7.5 mV/7.5 ms; ***$P < 0.001$ vs. the relevant control; $n$ = number of optic nerves.

Fig. 5. Normal P10 rat optic nerve ultrastructure is disrupted fol-
lowing co-exposure to 50 µM nicotine + 100 µM noradrenalin for 80
min followed by 30-min recovery. A: An oligodendroglial cell in control-
perfused nerve. The cell has classic features of a neonatal optic nerve
oligodendrocyte including narrow endoplasmic reticulum (arrow head),
numerous mitochondria (Mt), a clear cytoplasm, large nucleus with reg-
ular, unclumped chromatin. B: An astrocyte in control-perfused nerve
with classic features including wide-bore endoplasmic reticulum (arrow
head), dark bodies in the cytoplasm ("**"), and an irregular nucleus with
chromatic clumped under the envelope. Note in both "A" and "B" the
normal appearance of neighboring axons (Ax). C: Mean cell injury score
(see "Methods") for astrocytes and oligodendrocytes in control and 50
µM nicotine + 100 µM noradrenalin-treated nerves. ***$P < 0.001$ vs.
the relevant control value; **$P < 0.05$. Scale bars = 2 µm.
appeared to be unaffected by the exposure to the neurotransmitters and contained numerous microtubules and no evidence of axolemma breakdown or swelling, although the myelin of the few axons that had initiated myelination appeared damaged.

The ultrastructure of astrocytes in the optic nerves exposed to noradrenalin and nicotine was more profoundly affected than that of oligodendrocytes (see Fig. 7). Typical changes included the presence of very large and apparently swollen mitochondria, although these often retained an organized internal structure (Fig. 7A–C, long arrows). Membrane-delineated clear vacuoles were often seen (Fig. 7, asterisks), as well as swollen organelles, extensive regions of disintegrating cell membrane (Fig. 7, short arrows), and the presence of large areas of flocculent debris that appeared to be degenerating cell processes.

**DISCUSSION**

Previous studies have examined the acute effects of various neurotransmitters upon central white matter. Simmonds (1983) used grease-gap recording to demonstrate reproducible depolarization of rat optic nerve axons upon exposure to glycine or the GABA-A agonist muscimol. Sakatani et al. (1991, 1992, 1994) showed partial ablation of the compound action potential...
recorded from neonatal rat optic nerve following GABA-A receptor activation, an effect that was coupled to a rise in extracellular [K⁺] and axonal depolarization. A similar effect is seen in spinal dorsal column axons (Honmou et al., 1993; Lee et al., 1993), while GABA-B receptor activation is protective of the optic nerve against ischemic injury (Fern et al., 1994, 1995). nAChR activation also partially abolishes the compound action potential in the neonatal rat optic nerve (Zhang et al., 2004). In this case, nAChR activation produces Ca²⁺ influx into axons, although the effect upon the compound action potential is not dependent upon the Ca²⁺ influx and does not involve a large axonal depolarization (Zhang et al., 2004). nAChR activation can also modulate axonal excitability in mature myelinated axons of the thalamocortical pathway (Kawai et al., 2007). β-1 Adrenoreceptor activation increases the amplitude of submaximal compound action potentials in neonatal rat optic nerve in a Ca²⁺-dependent fashion, a phenomena that does not involve changes in extracellular [K⁺] (Honmou and Young, 1995). In all these studies, the period of receptor activation was relatively brief (<15 min), and the effects were largely reversible.

In the current study, 80-min perfusion with GABA or glycine suppressed the neonatal rat optic nerve compound action potential in a reversible fashion. In contrast, nAChR or adrenoreceptor activation for the same period had effects that were not reversed during a 30-min reperfusion period. The partial conduction failure induced by activation of nAChRs or adrenoreceptors was not associated with any clear axon pathology, and the axons retained numerous microtubules, neurofilaments, and mitochondria of a normal appearance. In contrast, widespread glial cell changes were evident and included a form of vacuolization of the oligodendrogial cell membrane and severe disruption of astrocytes. Astrocyte changes featured grossly enlarged mitochondria, generalized vacuolization, disintegration of processes, and breakdown of the cell membrane. A blinded count of cell injury showed significantly more astrocyte than oligo-
dendroglial cell damage. While many of the features of this injury are similar to those seen in glial cells following ischemia in this preparation (Thomas et al., 2004; Wilke et al., 2004), unique features included the presence of regional membrane vesiculation in oligodendroglial cells and the presence of expanded mitochondria retaining an internal structure in astrocytes. The absence of morphological changes in axons suggests that it is the glial injury that underlies the nonreversible conduction failure evoked by 80-min exposure to nAChR and adrenoreceptor activation. The absence of any significant increase in the level of conduction failure produced by co-activation of these receptors compared with the effects of a single agonist may indicate that a limit exists to the amount of conduction loss produced by acute glial injury. However, the extensive glial injury described here is likely to have further consequences for white matter function as reactive and delayed glial changes progress.

Glial cells are replete with neurotransmitter receptors. Cells of the oligodendrocyte lineage can express functional adrenoreceptor, nAChRs, GABA-A receptors, and glycine receptors (Aoki, 1992; Belachew and Gallo, 2004; Belachew et al., 1998, 2000; Berger et al., 1992; Cohen and Almazan, 1993; Gilbert et al., 1984; Khorchid et al., 2002a; Kirchhoff and Kettenmann, 1992; Oikawa et al., 2005; Pastor et al., 1995; Rogers et al., 2001; Takeda et al., 1995; Wang and Lidow, 1997). Astrocytes express a wide array of receptors, including nAChRs and adrenergic α-receptors capable of elevating intracellular Ca²⁺ (e.g., Duffy and MacVicar, 1995; Mantyh et al., 1995; Sharma and Vijayaraghavan, 2001). Astrocyte glycine receptor expression may be restricted to cells in the spinal cord (Kirchhoff et al., 1996; Pastor et al., 1995), while GABA-A receptor expression has been observed in a variety of astrocyte populations, including neonatal optic nerve astrocytes (Butt and Jennings, 1994). Glycine and GABA-A receptor activation evokes depolarization in neonatal astrocytes and cells of the oligodendroglial line due to the opening of a chloride conductance. The amplitude of the depolarization is unlikely to activate potential routes of Ca²⁺ influx such as Ca²⁺ channels or reverse Na⁺–Ca⁺ exchange, and there are no reports of elevations in glial Ca²⁺ following activation of either glycine or GABA-A receptors. Since nAChR activation can evoke Ca²⁺ rises in both astrocytes and oligodendroglia, while adrenergic α-receptors are documented to have the same effect at least in astrocytes, there is a good correlation between neonatal optic nerve injury and prolonged activation of neurotransmitter receptors that are capable of elevating Ca²⁺ in glia. Alternatively, adrenoreceptor overactivation can lead to glial injury following oxidative stress, although this occurs via delayed mechanisms (Khorchid et al., 2002b).

Microdialysis measurements show large ischemic rises in the extracellular concentration of GABA and glycine in central white matter (Shimada et al., 1993), and the current results indicate that these are unlikely to be damaging, although a chronic effect upon conduction is possible (Sakatani et al., 1993). While it is known that acetylcholine and adrenergic agonists such as noradrenaline are released into the extracellular space of the CNS during ischemia (e.g., Richards et al., 1993; Uchihashi et al., 1998; Yamamuro et al., 1996), recordings of resting and ischemic levels in brain white matter are lacking. The partial conduction block produced by adrenoreceptor antagonists in the current study indicates tonic receptor activation in white matter, and is consistent with the presence of an endogenous release mechanism potentially capable of elevating extracellular noradrenaline to toxic levels during ischemia.

Several observations suggest that the clinical significance of the toxic effects of nAChR and adrenoreceptor activation in central white matter may be high. The developmental disorder cerebral palsy is associated with white matter injuries that are thought to be ischemic in origin. The affected structures are likely to experience elevated levels of extracellular noradrenaline and acetylcholine during ischemia, either following release of endogenous pools of the neurotransmitters or indirectly from neighboring gray matter structures. Indeed, there is an association between maternal smoking and the incidence of perinatal white matter brain injury (e.g., Froen et al., 2002), while early exposure to nicotine can result in selective white matter abnormalities (e.g., Abdel-Rahman et al., 2005; Froen et al., 2002; Jacobsen et al., 2007). These effects are consistent with the elevated expression in developing central white matter structures of both nAChR (Swanson et al., 1987; Zhang et al., 2004) and adrenoreceptors (Dawidek and Robinson, 1993; Happe et al., 2004; Sanders et al., 2005). Other potential clinical links to the current findings include the noted absence of β2-adrenoreceptor expression in astrocytes of patients with multiple sclerosis (De Keyser et al., 2004). The current result showing that tonic β-adrenoreceptor activation is required if white matter function is to be maintained may therefore have relevance for white matter injury in postneonatal disorders such as multiple sclerosis.

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