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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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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. 80 min 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 non-reversible nerve failure indicating that tonic adrenoreceptor activation is required for nerve viability, while over activation 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 over-activation 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 signalling 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 signalling 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., McDonald et al., 1998; Fern and Moller 2000; Follett et al., 2000; Gallo and Ghiani 2000; Karadottir et al., 2005; Salter and Fern 2005; Micu et al., 2006), 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). We have recently shown functional glutamate uptake via high affinity transporters into central axons (Arranz et al., 2008), in addition to functional synaptic-type calcium channels and synaptic fusion type events in these axons during neonatal development (Alix et al., 2008). 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; Wilke et al., 2004; Salter and Fern 2005).

A number of neurotransmitters other than glutamate have been shown to have actions upon central white matter, in particular during neonatal development. Several studies reported endogenous GABA in the neonatal optic nerve (Lake 1992; Sakatani et al., 1992; Ochi et al., 1993), while GABA-A receptor activation produces a partial nerve block in this preparation (Sakatani et al., 1991; Sakatani et al., 1992). This effect is associated with extracellular $[K^+]$ accumulation and axon depolarization (Simmonds 1983; Sakatani et al., 1994). 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, adrenoceptor or GABA receptor activation upon neonatal rat optic nerve. nAChR or adrenoceptor activation were found to produce non-reversible injury characterized by partial conduction block and glial cell pathology. The results reveal the potential for non-glutamate 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 7-15 (referred to as "P10" throughout), placed in an interface perfusion chamber (Medical Systems, Greenvale, New York, USA), 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 (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 mOsmol 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 µsec 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: 800-10000 Hz.), digitized (25000 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 reported effects in isolated neonatal rat optic nerve (see Discussion).

Electron microscopy

Control nerves were perfused for 120 min with aCSF and were compared to nerves perfused for 80 min with 100 μ M noradrenalin + 50 μ M nicotine followed by 30 min washout using the standard protocol. Following perfusion, nerves were washed in Sorenson's buffer prior to post-fixation in 3% glutaraldehyde/Sorenson's for 90 min at room temperature. They were then washed in Sorenson's 3 x 5 min and stored at 5 $^{\circ}$ 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 envelop and had a darker cytoplasm, see Thomas et al., (2004) and (Vaughn 1969) for further details.

Statistics

Results are presented as mean $\pm$ SEM. Significance was determined by analysis of variance (ANOVA) using Tukey's post-test or by *t*-test, as appropriate.

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 $103.3 \pm 5.9\%$ of the initial value after 120 min of perfusion with aCSF; $n=5$; Fig 1 B, C). Perfusion with aCSF containing $100 \mu\text{M}$ of the inhibitory neurotransmitter glycine produced a gradual fall in compound action potential amplitude to $66.9 \pm 7.1\%$ at the end of the period of glycine perfusion ($P<0.005$ vs., control; $n=6$), recovering to $80.4 \pm 7.7\%$ upon washout (not statistically different from control, $P>0.05$). This effect was prevented by co-application of the selective glycine antagonist strychnine ($2 \mu\text{M}$; Fig 1 B). Perfusion with aCSF containing $100 \mu\text{M}$ of the inhibitory neurotransmitter GABA produced a more rapid fall in compound action potential amplitude to $82.9 \pm 7.4\%$ by the end of a standard 80 min perfusion period ($P<0.001$ vs., control; $n=7$; Fig 1 C, D). The compound action potential amplitude recovered to $91.4 \pm 7.9\%$ upon washout ($P>0.05$ vs., control). The effect of GABA upon the action potential was ablated by the GABA-A receptor antagonist picrotoxin ($100 \mu\text{M}$ added 10 min prior to exposure to GABA and washed out with the neurotransmitter; Fig 1 C, D).

Figure 1 near here

The cholinergic agonist nicotine ($50 \mu\text{M}$) evoked a gradual decline in the P10 optic nerve compound action potential (Fig 2 A, C). Action potential amplitude fell to $74.3 \pm 8.3\%$ after 80 min exposure ($P<0.005$ vs., control, $n=6$). Unlike the effect of

glycine and GABA, the fall in action potential amplitude evoked by nicotine was not reversed during a 30 min washout period (amplitude was $72.7 \pm 7.6\%$ of the initial value after washout; $P < 0.005$ vs., control). The cholinergic nicotinic antagonist mecamylamine blocked the effect of nicotine ($10 \mu\text{M}$ added 10 min prior to exposure to nicotine and washed out with the neurotransmitter; Fig 2 B, C).

Fig 2 near here

Perfusion with $100 \mu\text{M}$ of the adrenoreceptor agonist noradrenalin also resulted in a gradual decline in compound action potential amplitude, reaching $63.6 \pm 6.7\%$ of the initial value after 80 min ($n = 6$, $P < 0.001$ vs., control; Fig 3 A, B). As with nicotine, the fall in action potential amplitude evoked by noradrenalin was not reversed during a 30 min washout period (amplitude was $70.2 \pm 7.1\%$ of the initial value after washout; $P < 0.005$ vs., control). The effect of noradrenalin was not significantly reduced by the β -adrenoreceptor blocker propranolol ($10 \mu\text{M}$ added 10 min prior to exposure to noradrenalin and washed out with the neurotransmitter; Fig 3 B). Co-perfusion of propranolol and the α -adrenoreceptor antagonist phentolamine ($10 \mu\text{M}$ added as above), only served to increase the rate of action potential decline produced by noradrenalin (Fig 3 B). This curious result may be explained by the direct actions of adrenoreceptor blockers upon the compound action potential. Perfusion with $10 \mu\text{M}$ propranolol alone produced a fall in action potential amplitude to $69.1 \pm 6.9\%$ after 80 min exposure ($n = 7$; $P < 0.001$ vs., control; Fig 3 C). Washing out the β -adrenoreceptor antagonist resulted in action potential recovery to $86.0 \pm 11.0\%$ ($P > 0.05$ 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 3 C). However, co-application of propranolol + phentolamine resulted in incomplete recovery during washout ($73.1 \pm 8.9\%$; $P < 0.01$ vs., control). Block of tonic adrenoreceptor activation therefore resulted in a degree of non-reversible block of optic nerve conduction, while over-activation 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 non-reversible 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 3 b).

Fig 3 near here

Nicotine or noradrenalin applied alone produced a largely non-reversible 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 4 A for 50 μ M nicotine, 100 μ M noradrenalin, and 50 μ M nicotine + 100 μ M noradrenalin. The effect of co-exposure upon compound action potential amplitude is shown in Fig 4 B, revealing a $64.0 \pm 4.8\%$ 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 4 C, revealing no significant difference between them. This may indicate a common mode of action between these conditions.

Fig 4 near here

To examine the nature of the non-reversible effects of co-application of 50 μ M nicotine + 100 μ M noradrenalin, 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 5 A, 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 5 C). The level of injury was significantly greater in astrocytes compared to oligodendroglia in the treated nerves ($P < 0.005$). Typical oligodendroglia 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 breakdown of the cell membrane could also be observed in oligodendrocyte processes engaged in ensheathing and myelinating axons (e.g., Fig 6 B: "*"). Axon cylinders 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.

Figs 5-6 near here.

The ultrastructure of astrocytes in the optic nerves exposed to noradrenalin and nicotine was more profoundly affected than that of oligodendrocytes (Fig 7). Typical changes included the presence of very large and apparently swollen mitochondria, although these often retained an organized internal structure (Fig 7 A-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.

Fig 7 near here

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 Ca^{2+} influx into axons, although the effect upon the compound action potential is not dependent upon the Ca^{2+} 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 sub-maximal

compound action potentials in neonatal rat optic nerve in a Ca^{2+} -dependent fashion, a phenomena that does not involve changes in extracellular $[\text{K}^+]$ (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 oligodendroglial 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 oligodendroglial 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 non-reversible 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 to 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 (Gilbert et al., 1984; Aoki 1992; Kirchhoff and Kettenmann 1992; Berger et al., 1992; Cohen and Almazan 1993; Takeda et al., 1995; Pastor et al., 1995; Wang and Lidow 1997; Belachew et al., 1998; Belachew et al., 2000; Rogers et al., 2001; Khorchid et al., 2002a; Belachew and Gallo 2004; Oikawa

et al., 2005). Astrocytes express a wide array of receptors, including nAChRs and adrenergic α -receptors capable of elevating intracellular Ca^{2+} (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 (Pastor et al., 1995; Kirchhoff et al., 1996), 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 Ca^{2+} influx such as Ca^{2+} channels or reverse Na-Ca exchange, and there are no reports of elevations in glial Ca^{2+} following activation of either glycine or GABA-A receptors. Since nAChR activation can evoke Ca^{2+} 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 Ca^{2+} in glia. Alternatively, adrenoreceptor over activation can lead to glial injury following oxidative stress, although this occurs via delayed mechanisms (Khorchid et al., 2002b).

Micro-dialysis 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 noradrenalin are released into the extracellular space of the CNS during ischemia (e.g., Richards et al., 1993; Yamamuro et al., 1996; Uchihashi et al., 1998), 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 noradrenalin 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 noradrenalin and acetylcholine during ischemia, either following release of endogenous pools of the neurotransmitters or indirectly from neighbouring grey 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 β_2 -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 post-neonatal disorders such as multiple sclerosis.

Figure legends

Figure 1

Glycine or GABA-A receptor activation has reversible effects upon the compound action potential recorded from the P10 rat optic nerve. A: Compound action potentials recorded before (left), during (middle) and after (right) 80 min of perfusion with 100 μ M glycine (dashed lines are for comparison purposes). B: The data is plotted for nerves perfused with aCSF throughout ("control"), those exposed to glycine and those exposed to glycine + strychnine. C: Compound action potentials recorded at T = 0 min (arrow) and after perfusion with either: aCSF (left), 100 μ M GABA (middle) or 100 μ M GABA + 100 μ M picrotoxin (right) for 80 min (picrotoxin applied 10 min prior to GABA and washout out with GABA). D: Data plot showing that the compound action potential amplitude declines upon exposure to GABA, an effect that is blocked by picrotoxin. Note that the effects of both GABA and glycine are reversed during the 30 min washout period at the end of the experiment. Error bars are SEM, scale bars = 7.5 mV / 7.5 msec.

Figure 2

nAChR activation has non-reversible effects upon the compound action potential recorded from the P10 rat optic nerve. A: Compound action potentials recorded before (left), during (middle) and after (right) 80 min of perfusion with 50 μ M nicotine. Note the decline in the action potential amplitude in nicotine and the absence of recovery following washout. B: Compound action potentials recorded before (arrows) and after an 80 min period of perfusion with aCSF (left), 50 μ M nicotine (middle) or 50 μ M nicotine + 10 μ M of the nAChR blocker mecamylamine (right). C: The data is plotted showing the non-reversible decline in action potential amplitude in nicotine, and the block of this effect by mecamylamine. Error bars are SEM, scale bars = 7.5 mV / 7.5 msec.

Figure 3

β -adrenergic receptor activation has non-reversible effects upon the compound action potential recorded from the P10 rat optic nerve. A: Compound action potentials recorded before (left), during (middle) and after (right) 80 min of perfusion with 100 μ M noradrenalin. Note the decline in the action potential amplitude in noradrenalin and the absence of recovery following washout. B: Data plot showing the non-reversible decline in action potential amplitude produced by 100 μ M noradrenalin, and the absence of block of this effect by 10 μ M of the β -adrenergic blocker propranolol or 10 μ M of the α -adrenergic phentolamine. C: Data plot showing that while perfusion with 10 μ M phentolamine had no significant effect upon the action potential, 10 μ M propranolol reduced compound action potential amplitude. There was no additive effect of co-application with both blockers upon the decline observed in the presence of propranolol alone, but incomplete recovery was found following perfusion with both blockers. Error bars are SEM, scale bars = 7.5 mV / 7.5 msec.

Figure 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 μ M nicotine, 100 μ M noradrenalin, or 50 μ M nicotine + 100 μ M noradrenalin. B: Data plot showing the non-reversible 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 μ M nicotine, 100 μ M noradrenalin, or 50 μ M nicotine + 100 μ M noradrenalin. Error bars are SEM, scale bars = 7.5 mV / 7.5 msec, "****" = $P < 0.001$ vs., the relevant control, 'n' = number of optic nerves.

Figure 5

Normal P10 rat optic nerve ultrastructure is disrupted following co-exposure to 50 μ M nicotine + 100 μ M noradrenalin for 80 min followed by 30 min recovery. A: An oligodendroglial cell in control perfused nerve. The cell has classic features of an neonatal optic nerve oligodendrocyte including narrow endoplasmic reticulum (arrow head), numerous mitochondria (Mt), a clear cytoplasm, large nucleus with regular, 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 chromatin clumped under the envelope. Note in both "A" and "B" the normal appearance of neighbouring axons (Ax). C: Mean cell injury score (see Methods) for astrocytes and oligodendrocytes in control and 50 μ M nicotine + 100 μ M noradrenalin treated nerves. "****" = $P < 0.001$ vs., the relevant control value, "***" $P < 0.05$. Scale bars = 2 μ m.

Figure 6

Features of oligodendrocyte changes induced by co-exposure to 50 μ M nicotine + 100 μ M noradrenalin for 80 min followed by 30 min recovery. A: An oligodendrocyte (Oli) showing mitochondria that appear normal and regions where the cell membrane has broken down into vacuoles (box shown in greater detail on the right). Membrane breakdown features formation of numerous vacuoles of various sizes ("*"), and is focal with neighbouring region of cell membrane appearing intact (short arrows). B: Similar changes are evident in this oligodendrocyte (box expanded to the right). Note regions of cell membrane (short arrows) between regions of vacuolization. Similar changes are apparent in an oligodendrocyte process that is myelinating a near-by axon ("*"), although the axon itself and the other axons in the micrograph appear normal (Ax). The myelin sheath of larger axons in both "A" and "B" appear disrupted (mAx). C: Two closely apposed oligodendrocytes exhibit extensive cell membrane vacuolization (box expanded to the right). Scale bars = 2 μ m.

Figure 7

Features of astrocyte changes induced by co-exposure to 50 μ M nicotine + 100 μ M noradrenalin for 80 min followed by 30 min recovery. A: An astrocyte (Ast) neighboured by two oligodendrocytes exhibits severe cellular disruption that includes the presence of swollen and expended mitochondria (Mt) and the presence of numerous cytoplasmic vacuoles ("*"). The boxed area is shown in greater detail to the right. Note the normal appearance of near-by axons (Ax). B: This degenerating astrocyte has extremely large mitochondria in the cytoplasm (box expanded to the right). Note the presence of cristae within these structures. C: Neighbouring astrocytes have lost membrane integrity (short arrows), and have regions filled with swollen organelles and clear vacuoles ("*"). Boxed area expanded to the right. Scale bars = 2 μ m in B-C and 5 μ m in A.

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Glycine or GABA-A receptor activation has reversible effects upon the compound action potential recorded from the P10 rat optic nerve. A: Compound action potentials recorded before (left), during (middle) and after (right) 80 min of perfusion with 100 μ M glycine (dashed lines are for comparison purposes). B: The data is plotted for nerves perfused with aCSF throughout ("control"), those exposed to glycine and those exposed to glycine + strychnine. C: Compound action potentials recorded at T = 0 min (arrow) and after perfusion with either: aCSF (left), 100 μ M GABA (middle) or 100 μ M GABA + 100 μ M picrotoxin (right) for 80 min (picrotoxin applied 10 min prior to GABA and washout out with GABA). D: Data plot showing that the compound action potential amplitude declines upon exposure to GABA, an effect that is blocked by picrotoxin. Note that the effects of both GABA and glycine are reversed during the 30 min washout period at the end of the experiment. Error bars are SEM, scale bars = 7.5 mV / 7.5 msec.

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Figure 2 nAChR activation has non-reversible effects upon the compound action potential recorded from the P10 rat optic nerve. A: Compound action potentials recorded before (left), during (middle) and after (right) 80 min of perfusion with 50 μ M nicotine. Note the decline in the action potential amplitude in nicotine and the absence of recovery following washout. B: Compound action potentials recorded before (arrows) and after an 80 min period of perfusion with aCSF (left), 50 μ M nicotine (middle) or 50 μ M nicotine + 10 μ M of the nAChR blocker mecamylamine (right). C: The data is plotted showing the non-reversible decline in action potential amplitude in nicotine, and the block of this effect by mecamylamine. Error bars are SEM, scale bars = 7.5 mV / 7.5 msec. 101x135mm (250 x 250 DPI)

Figure 3 β -adrenergic receptor activation has non-reversible effects upon the compound action potential recorded from the P10 rat optic nerve. A: Compound action potentials recorded before (left), during (middle) and after (right) 80 min of perfusion with 100 μ M noradrenalin. Note the decline in the action potential amplitude in noradrenalin and the absence of recovery following washout. B: Data plot showing the non-reversible decline in action potential amplitude produced by 100 μ M noradrenalin, and the absence of block of this effect by 10 μ M of the β -adrenergic blocker propranolol or 10 μ M of the α -adrenergic phentolamine. C: Data plot showing that while perfusion with 10 μ M phentolamine had no significant effect upon the action potential, 10 μ M propranolol reduced compound action potential amplitude. There was no additive effect of co-application with both blockers upon the decline observed in the presence of propranolol alone, but incomplete recovery was found following perfusion with both blockers. Error bars are SEM, scale bars = 7.5 mV / 7.5 msec.

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Figure 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 μ M nicotine, 100 μ M noradrenalin, or 50 μ M nicotine + 100 μ M noradrenalin. B: Data plot showing the non-reversible 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 μ M nicotine, 100 μ M noradrenalin, or 50 μ M nicotine + 100 μ M noradrenalin. Error bars are SEM, scale bars = 7.5 mV / 7.5 msec, "****" = $P < 0.001$ vs., the relevant control, 'n' = number of optic nerves.

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Figure 5 Normal P10 rat optic nerve ultrastructure is disrupted following co-exposure to 50 μ M nicotine + 100 μ M noradrenalin for 80 min followed by 30 min recovery. A: An oligodendroglial cell in control perfused nerve. The cell has classic features of an neonatal optic nerve oligodendrocyte including narrow endoplasmic reticulum (arrow head), numerous mitochondria (Mt), a clear cytoplasm, large nucleus with regular, 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 envelop. Note in both "A" and "B" the normal appearance of neighbouring axons (Ax). C: Mean cell injury score (see Methods) for astrocytes and oligodendrocytes in control and 50 μ M nicotine + 100 μ M noradrenalin treated nerves. "****" = $P < 0.001$ vs., the relevant control value, "***" $P < 0.05$. Scale bars = 2 μ m.

Figure 6 Features of oligodendrocyte changes induced by co-exposure to 50 μ M nicotine + 100 μ M noradrenalin for 80 min followed by 30 min recovery. A: An oligodendrocyte (Oli) showing mitochondria that appear normal and regions where the cell membrane has broken down into vacuoles (box shown in greater detail on the right). Membrane breakdown features formation of numerous vacuoles of various sizes ("*"), and is focal with neighbouring region of cell membrane appearing intact (short arrows). B: Similar changes are evident in this oligodendrocyte (box expanded to the right). Note regions of cell membrane (short arrows) between regions of vacuolization. Similar changes are apparent in an oligodendrocyte process that is myelinating a near-by axon ("*"), although the axon itself and the other axons in the micrograph appear normal (Ax). The myelin sheath of larger axons in both "A" and "B" appear disrupted (mAx). C: Two closely apposed oligodendrocytes exhibit extensive cell membrane vacuolization (box expanded to the right). Scale bars = 2 μ m.

Figure 7 Features of astrocyte changes induced by co-exposure to 50 μ M nicotine + 100 μ M noradrenalin for 80 min followed by 30 min recovery. A: An astrocyte (Ast) neighbored by two oligodendrocytes exhibits severe cellular disruption that includes the presence of swollen and expended mitochondria (Mt) and the presence of numerous cytoplasmic vacuoles ("*"). The boxed area is shown in greater detail to the right. Note the normal appearance of near-by axons (Ax). B: This degenerating astrocyte has extremely large mitochondria in the cytoplasm (box expanded to the right). Note the presence of cristae within these structures. C: Neighbouring astrocytes have lost membrane integrity (short arrows), and have regions filled with swollen organelles and clear vacuoles ("*"). Boxed area expanded to the right. Scale bars = 2 μ m in B-C and 5 μ m in A.