# Picrotoxin ($GABA_A$ receptor antagonist) shows a protective role in brain injury during neonatal development

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## Abstract

$GABA_A$-receptor antagonist picrotoxin has been shown to have actions upon brain injury during neonatal development. The significance of the expression of $GABA_A$-receptors in developing brain specifically white matter during injury has not been completely examined. As well previous studies have concentrated upon brief periods of receptor activation and later points in development. For this paper work, the injury capacity of a standard 90-min period of both oxygen-glucose deprivation (OGD) and artificial cerebrospinal fluid (aCSF) co-perfused with a $GABA_A$-R antagonist were examined using electrophysiology and ultrastructural analysis techniques of P0 rat optic nerves (RONs) (a model of non-myelinated brain white matter). The result reveals the potential role of inhibitory other than excitatory neurotransmitters mediated injury in young brain in early points of development. It shows that $GABA_A$-R block both increased compound action potential (CAP) under control conditions, and protected the RONs from OGD-induced injury. The protective effects of 100μM $GABA_A$-R antagonist against OGD-induced axonal injury in P0-RONs using electrophysiological technique is consistent with ultra-micrograph data presented here showing protective effects against OGD-induced axonal and glial injury in P0-RONs. The results of both electrophysiology and microscopy are consistent with a potential role of $GABA_A$-R-mediated injury in neonatal brain. *This indicates the protective role of the $GABA_A$-R antagonist against ischaemic injury in non-myelinated brain.*

***Key words:*** *neonatal brain, Ischaemia, Picrotoxin, electrophysiology and electron microscopy*

## Introduction

### Ɣ-amino-butyric acid (GABA)

GABA is the major transmitter for rapid inhibitory synaptic transmission in the brain (Olsen and DeLorey, 1999). It was discovered in the 1950s by Eugene Roberts and J. Awapara. It is synthesised by a specific enzyme, glutamic acid decarboxylase (GAD), from L-glutamate (Lee *et al.,* 2011) (*see Fig 1)*. Cultured human astrocytes express the mRNA and proteins for the GABA synthesising enzyme (GAD), the GABA metabolising enzyme GABA-transaminase (GABA-T), and the $GABA_A$, and $GABA_B$ receptors (Lee *et al.*, 2011). Cultured microglia does not express GAD, but they do express GABA-T and the $GABA_A$ and $GABA_B$ receptors (Lee *et al.*, 2011).

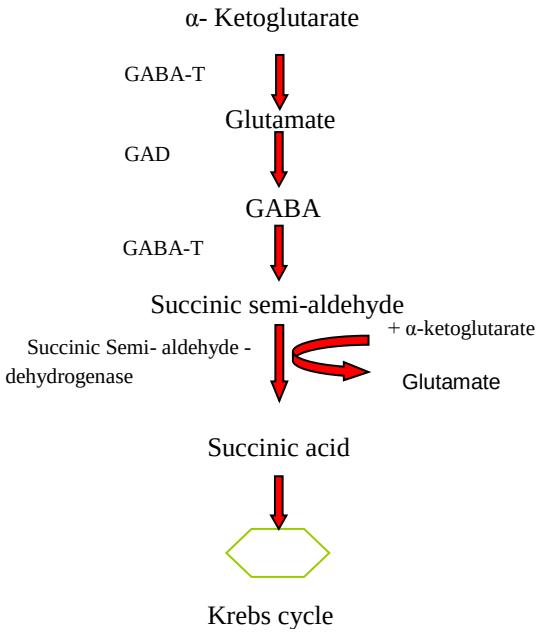



**Figure -1: GABA synthesis and metabolism.**

GABA is synthesised from α-ketoglutarate, which is formed from glucose metabolism via the Krebs cycle. The α-ketoglutarate is transaminated by the enzyme GABA-T into glutamate. #Glutamate is metabolised by the GAD enzyme to GABA, which is consequently metabolised to succinic semi-aldehyde by the enzyme GABA-T. Succinic semi-aldehyde in combination with α-ketoglutarate results in the formation of glutamate, though succinic semi-aldehyde might be metabolised to succinic acid which is required in the Krebs cycle (adapted from Erlander and Tobin, 1991; Paul, 2000).

These findings indicate that astrocytes have GABAergic properties, while microglias have GABAceptive properties (Lee *et al.,* 2011). Reports suggest that astrocytes express multiple receptors designed to facilitate GABA release through $Ca^{2+}$-dependent and $Ca^{2+}$-independent mechanisms (Anderson and Swanson, 2000; Hamilton and Attwell, 2010). Heja *et al.* (2009) showed that the receptor-mediated release of GABA is a $Ca^{2+}$-dependent mechanism, whereas GABA release from astrocytes by GABA-transporters is a $Ca^{2+}$-independent mechanism

which may be coupled with glutamate release. The $Ca^{2+}$-dependent release of GABA has been demonstrated in the adult brain, with less evidence in the developing brain (Saransaari and Oja, 1997; Kontro and Oja, 1987). During the neonatal period, GABA release is entirely $Ca^{2+}$ independent, and becomes $Ca^{2+}$-dependent at later stages of development (Balcar *et al.*, 1986). However, GABA release is markedly affected by $Na^+$ and $Cl^-$ removal, indicating that GABA release in ischaemia may be mediated partially by $Na^+$ and $Cl^-$ -channels (Saransaari and Oja, 1992; Bernath, 1992). This anion-channel-mediated release is probably attributable to cell swelling and leakage through disordered membrane structures (Saransaari and Oja, 2008). GABA release during ischaemia has been suggested to be either neuroprotective by inducing the hyperpolarisation of neurones or reducing the release of the excitotoxic neurotransmitter glutamate, or to be neurotoxic by enhancing $Cl^-$ influx into neurones that contribute to cellular swelling and neuronal death (Allen *et al.*, 2004). GABA is an endogenous agonist for the three subtypes of GABA receptors; $GABA_A$, $GABA_B$, and $GABA_C$ (Chebib and Johnston, 1999). $GABA_A$-Rs are one of the large superfamilies of ligand-gated ion channels which include inotropic glutamate receptors (iGluRs), nicotinic-cholinergic receptors and glycine receptors (Barnard *et al.*, 1987; Squires *et al.*, 1983). $GABA_A$-Rs are expressed on cells of oligodendrocyte lineage (Aoki, 1992; Belachew and Gallo, 2004), and on the astrocytes of neonatal optic nerves (Butt and Jennings, 1994). Immunohistochemical studies conducted on mouse brains have provided growing anatomical evidence that white matter interstitial cells comprise GABAergic interneurons (Engelhardt *et al.*, 2011). Determining the pharmacology of GABA-Rs may lead to the development of novel therapies for use in CNS ischaemia (Costa *et al.*, 2004). It has been reported that cerebral ischaemia is the major cause of death worldwide, and the concentration of inhibitory and excitatory is increased during ischaemia (Hutchinson *et al.*, 2002). The role of the excitatory neurotransmitters and their receptors has been extensively studied during ischaemia, but the role of the inhibitory amino acid GABA and its receptors is less well established (Choi, 1992). A few investigators have focused on the role of GABA in the pathogenesis of neuronal death during ischaemia (Choi, 1992). And t3ghe significance of the expression of GABA-Rs in developmental central white matter reveals the potential role of these receptors in mediating ischaemic injury in neonates (Constantinou and Fern, 2009). Picrotoxin and t-butylbicyclophosphorothionate (TBPS) are non-competitive $GABA_A$-R antagonists; they do not bind directly to the recognition site on GABA-R, but to a separate recognition site within the receptor complex (Squires *et al.*, 1983). Picrotoxin has a protective role in brain ischaemia in neonate (Hutchinson *et al.*,

2002). It's a GABA-gated $Cl^-$--channel blocker that prevents the influx of $Cl^-$- through GABA-gated $Cl^-$--channels (Inglefield and Schwartz-Bloom, 1998).

**Methods**

Perinatal rats were used at P0, an age when the optic nerve is non-myelinated. RONs are considered to be an ideal model to investigate the function of CNS and the mechanism of injury, because they are completely white matter tracts, and they are devoid of the complications of neuronal cell bodies, the retinal ganglion cells (Ransom et al., 1997). Optic nerves were dissected between the optic disc and optic chiasm and then placed in aCSF. All dissected nerves were placed in an interface perfusion chamber *where* they were allowed to equilibrate for an hour before being inserted into the electrodes. This was done to permit the RON time to recover from the trauma of the dissection and so recover homeostatic control. The RONs were perfused with aCSF at a rate of 1-2ml/min. The temperature was maintained at 37˚C throughout the time of the experiment (101C temp controller, Warner etc.), and the RONs oxygenated with 95%$O_2$/5%$CO_2$, which was passed through aCSF and through a water chamber for warming and humidification. Electrophysiology was used to record CAPs in this work. Upon starting an experiment, CAP recordings were made under control conditions (normal aCSF and 95%$O_2$/5%$CO_2$) for the first 10 min to ensure an environment conducive to stable recording had been achieved. To simulate ischaemia RONs were exposed to a perfusate which had been continuously bubbled with a 95%$N_2$/5%$CO_2$ mixture and from which glucose was omitted for 90 min. Following OGD, exposure of RONs to normal aCSF for 90 min was allowed, during this time the RON achieves its maximum functional recovery. When the experiment solutions and gases were changed, it took about 2 min for the new solutions to reach the interface perfusion chamber, this time recorded as the time of the solutions and gases were physically swapped. It has been shown by previous studies using a similar experimental design that the gaseous environment changes from normoxic to anoxic (and vice versa) in approximately 2 min (Stys *et al.,* 1990; Stys *et al.,* 1992). Observations were made from electrical stimulus evoked CAPs using glass electrodes. The relationship between increasing stimulus intensity and the CAP is proportional. Increasing the stimulus current will result in an increase in the CAP up to maxima (James *et al.,* 2010). CAPs for nerves younger than P15 were elicited by square constant-voltage stimulus pulses of 150-600 μsec duration which were delivered by an isolated stimulus unit at one minute intervals (unless otherwise stated). The CAPs were elicited by 125% supra-maximal stimuli applied to the proximal end of the RON by stimulating electrode. The recorded signal was amplified ten times, filtered at

800Hz (low pass) in experiments using immature RONs a period of 40 milliseconds every 60 seconds (unless otherwise stated). The data were transferred to computer where it was exported to excel and analysed using Signal software (Cambridge Electronic Design Ltd 2002). CAP loss is an indication for a loss of axonal function (Fern *et al.*, 1998), which is well correlated with pathological changes in the axonal structures such as, loss of axonal integrity, mitochondrial damage, dissolution of microtubules and neurofilaments (Alix and Fern, 2009). The amplitude of the biphasic CAP, calculated from the maxima of the positively deflected peak minus the minima of the negatively deflected peak, was used as a measure of the functional integrity of neonatal RONs (Fern *et al.*, 1998). Attempts to use the area of the CAP as in adult were not made due to interference from the stimulus artefact.

Data are expressed as mean ± SEM, significance determined by Bonferroni`s Multiple Comparison Test or ANOVA as appropriate. Ultrastructure was examined to show the axonal and glial injury in P0 RONs following regular OGD, OGD+picrotoxin.

**Result**

**GABAA-R antagonist (100µM picrotoxin) during OGD and control**

When OGD was performed in the presence of 100µM picrotoxin, the CAP fell to 39.3 ± 5.7% of the initial value at the end of OGD and recovered significantly following reperfusion with normal conditions to 72.1 ± 3.6% of the initial value, (n=8; P<0.0001 vs OGD); *(see Fig 2).* Application of OGD was preceded by 10 min aCSF co-perfused with 100µM picrotoxin, and the CAP increased up to 117 ± 0.04% of the initial value. Optic nerves were exposed to aCSF co-applied with 100µM picrotoxin for 30 min period after OGD and before re-introduction of normal conditions, during this time; the CAP increased to 80.01 ± 3.1% of the initial value; *(see Fig 2).* The CAP gradually increased within the first 20-30 min during aCSF co-perfused with 100µM picrotoxin and reached 148.8 ± 14.2% of the initial value, then declined to 75.7 ± 3.7% of the initial value at the end of 90 min of perfusate. After this, the CAP dropped gradually following restoration of normal conditions to 46.9 ± 12.5% of the initial value, (n=8; P<0.0001 vs control).

Another regime was performed during aCSF co-applied with 100µM picrotoxin; P0-RONs were exposed to the perfusate for 180 min without re-instruction of normal conditions. A continuous perfusion with 100µM picrotoxin produced a gradual increase in the CAP amplitude to 195.7 ± 5.5% of the initial value at the end of the experiment, (n=8; P<0.0001 vs control); *(see Fig 3).* The result shows that $GABA_A$-R block both increased CAP under control

conditions, and protected the RON from OGD-induced injury. *This indicates the protective role of the $GABA_A$-R antagonist against ischaemic injury in pre-myelinated axons.*

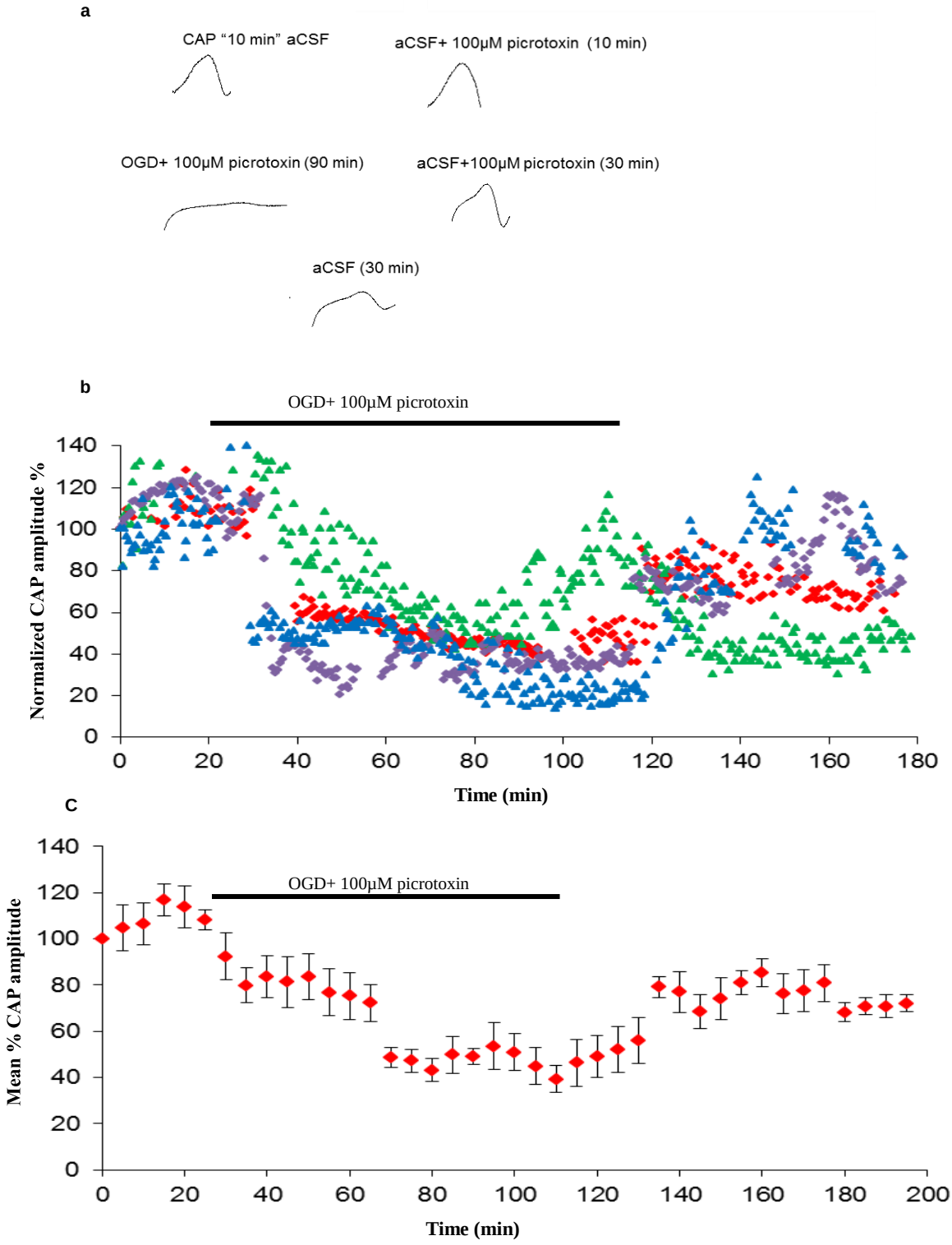
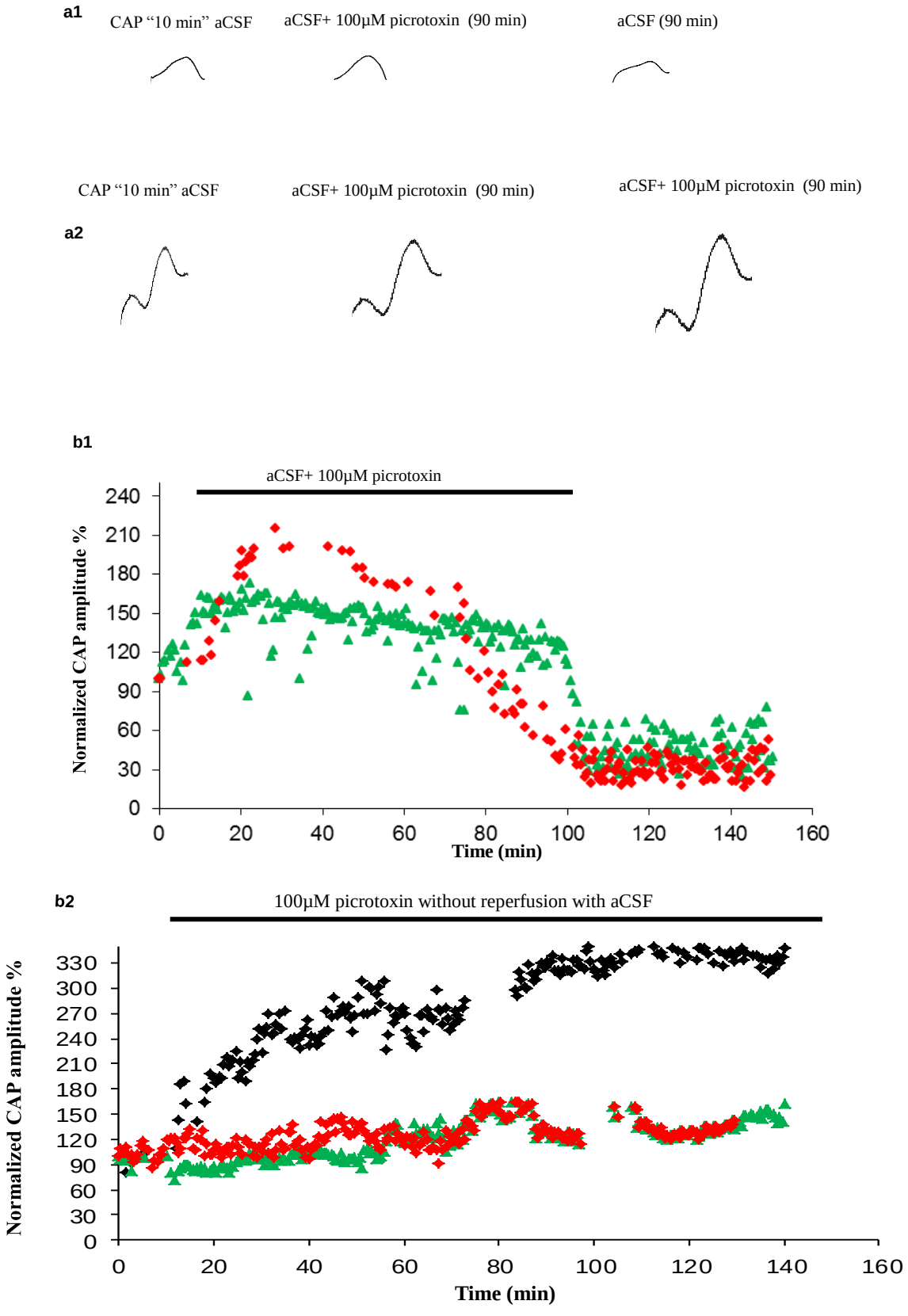



**Figure- 2: Application of $GABA_A$-R antagonist picrotoxin (100µM) during 90 min OGD evoked a partial decline in the CAP in a reversible fashion in P0-RONs.**

**a**, A representative CAP during normal conditions for 10 min, during the first 10 min exposure to aCSF+100µM picrotoxin, during exposure to OGD+ 100µM picrotoxin for 90 min, during re-perfusion with aCSF+100µM picrotoxin for 30 min and during re-instruction of normal

conditions for 30 min. **b**, Plot of four single experiments against time. Red plot is an example of the CAP in (a). **c** , Mean data showing the changes in the CAPs before, during OGD-co-applied with 100µM picrotoxin, and after restoring the normal conditions, n=8. CAP is normalized to 100% at zero time. CAP at the end of OGD is (39.3± 5.7); CAP after restoring the normal conditions is (72.1± 3.6). Error bars are SEM.



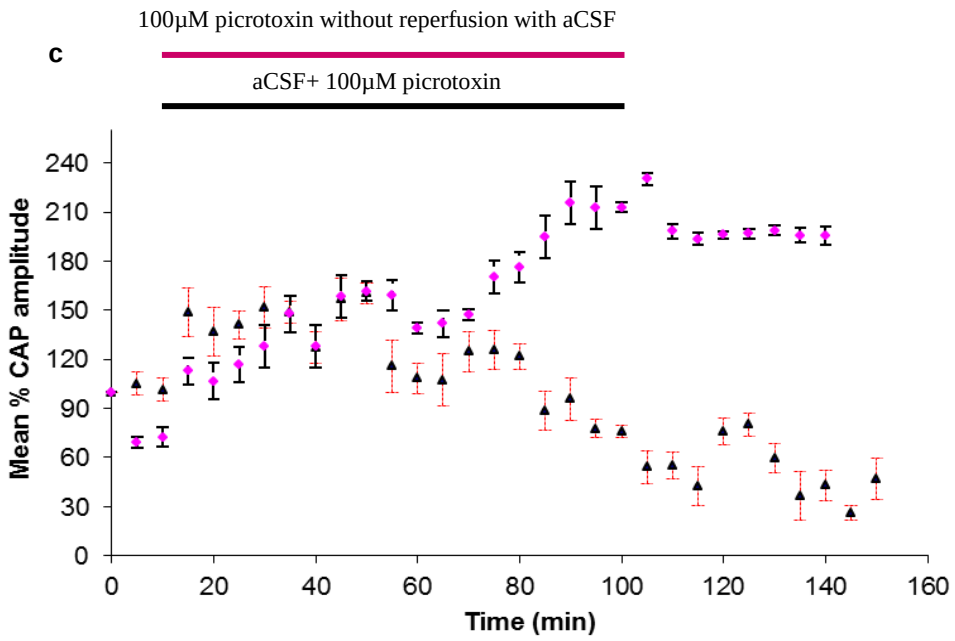



**Figure -3: Persistent exposure to $GABA_A$-R antagonist picrotoxin (100µM) blocks the irreversible decline in CAP in P0- RONs.**

**a1,** CAPs recorded before, during, and after 90 min of perfusion with aCSF+100µM picrotoxin. **a2**, representative CAPs during normal conditions and 180 min of continuous perfusion with 100µM picrotoxin. **b1**, plots of three experiments showed the changes in the CAP during exposure to 90 min of aCSF+100µM picrotoxin and after reperfusion with normal conditions. **b2**, Plots of three experiments showed the changes in the CAP during continuous exposure to aCSF+100µM picrotoxin. **c**, Mean plot showing the irreversible decline in the CAPs after restoring the normal conditions (black plots, n=8), an effect that is blocked by continuous exposure to aCSF+100µM picrotoxin (pink plots, n=8). CAP is normalized to 100% at zero time, CAP at the end of 90 min exposure to aCSF+100µM picrotoxin is (75.7± 3.7), and after reperfusion with normal conditions is (46.9± 12.5), CAP during continuous perfusion with aCSF+100µM picrotoxin is (195.7± 5.5). Error bars are SEM.

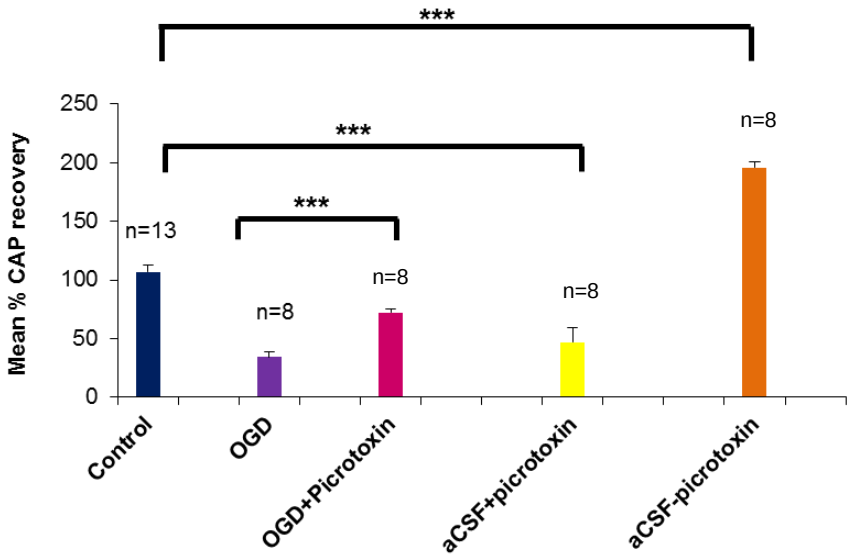



**Figure-4: Summary of changes in the CAP amplitude of P0-RONs during exposure to $GABA_A$-R antagonist in P0-RONs.**

Histogram summarized the protective effect of $GABA_A$-R antagonist in control conditions and OGD. It showed a large recovery in the CAPs in both situations (OGD- & aCSF- picrotoxin (100µM) without re-perfusion with normal conditions). ***=p<0.0001

Electron microscopy was used to illustrate the ultra-structural changes to the axons as well as to the glia evoked by OGD, and the role of GABA-R blocker picrotoxin in protecting the immature brain structures.

P0-RONs was analysed in cross-sections; axons were readily identified by the presence of axolemma, microtubules and a cylindrical structure. Axons were generally found in clusters embedded within fields of less electron-dense and fewer cylindrical processes that either contained few tubular or filamentous elements, or glial filaments, identifying them as astrocyte processes *(see Fig5).* Astrocytes and occasional glioblasts populated the underlying intermediate zone where axons are observed in the cross-sections of the electron-microscope with an axon diameter of ≤ 500 nm Astrocytes contain obvious mitochondria, endoplasmic reticulum, Golgi apparatus and glycogen particles. Blinded counting of axonal density showed a high number of identifiable axons in control nerves (mean=11±0.737 axons/$\mu^2$; n=1949 axons/13 sections form 3 nerves). Viability scoring showed that the majority of axons have a score of "3", which correlates to no pathology (mean= 2.9±0.09; n=1949/12 sections). Viability scoring of glia in P0-RONs showed that the majority of glia also had a score of "3" (mean= 2.9±0.05; n=24/16 sections).

Axon injury in nerves exposed to 90 min OGD was highly variable, with some axons showing axoplasmic and mitochondria swelling, the presence of flocculent debris in the axon cylinder, and loss of microtubules *(see Fig5).* Glia injury during 90 min OGD was also significant *(see Fig6).* The cells appeared swollen and showed severely disrupted processes, and the membrane integrity was lost in many cases. Blinded counting of axonal density showed a significantly lower number of identifiable axons following OGD (mean= 2.7±0.74 axons/$\mu^2$; n=300 axons/7 sections in 3 nerves; $P<0.0001$ vs control perfused RONs). Blinded axonal viability scores were significantly lower in identifiable axons following OGD; most scored 1.5 (mean= 1.7±0.31; $P<0.0001$ vs control perfused RONs) *(see Fig5).* Blinded assessment of astrocyte injury following OGD resulted in an extremely low score of "1" in many cells (mean= 1.0±.018; n=30/13 sections, 3 nerves; $P<0.001$ vs control perfused RONs) *(see Fig6).*

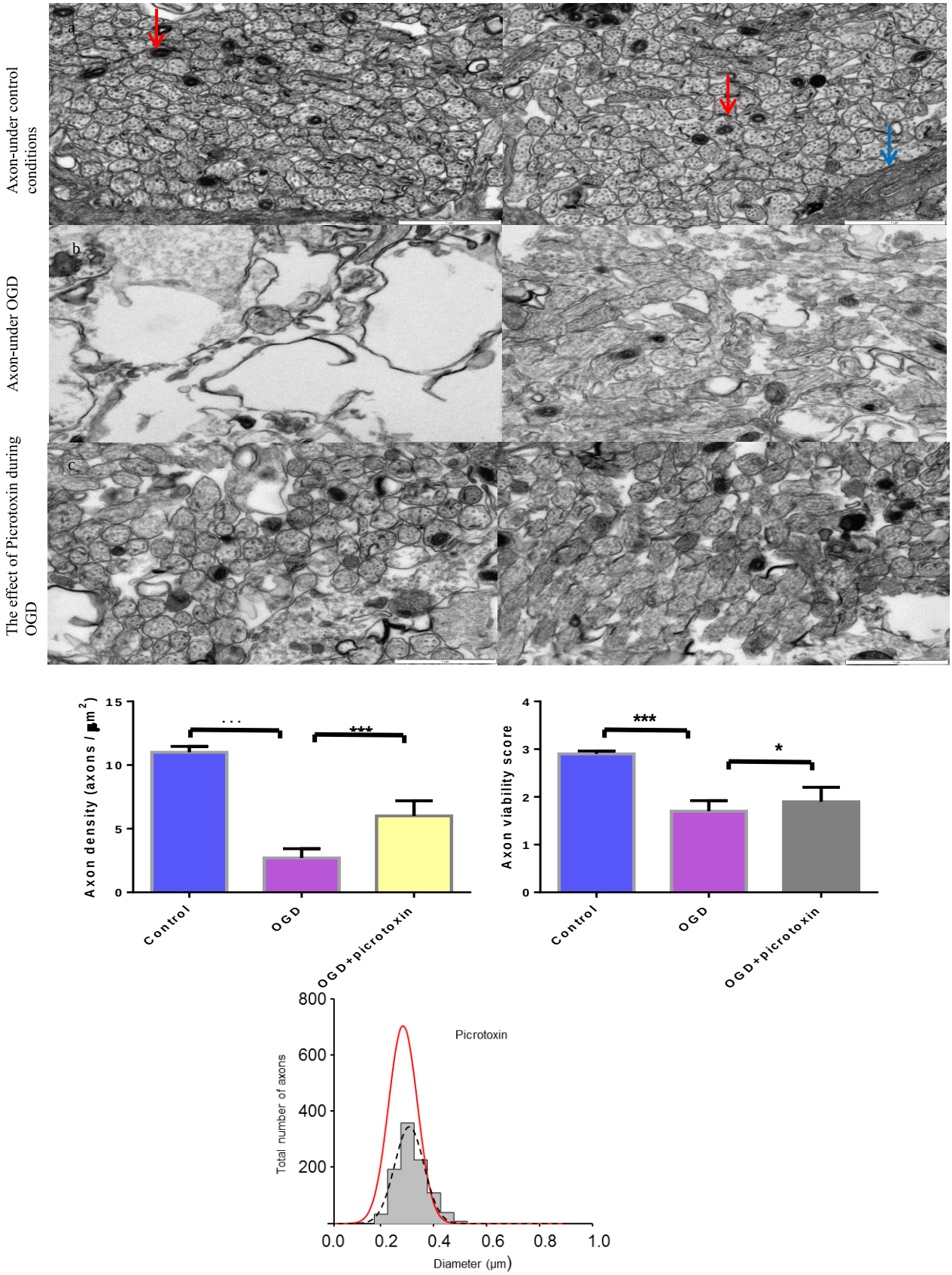



**Figure-5**: **GABA-R block picrotoxin (100µM) is protective against OGD-induced axonal injury in P0-RONs.**

**a,** Representative ultra-micrographs of axons from control. Normal axons (red arrows) and astrocyte processes (blue arrow). **b,** OGD (90 min)-induced axonal injury. **c,** Representative ultra-micrographs of axons from OGD + picrotoxin (90 min). **d,** Picrotoxin produced significant protection against OGD-induced axonal density damage and less significant effect of protection against OGD-induced axonal injury;***P<0.0001& *P<0.01respectively. Error bars represent SEM. **e,** Total number of the axons during OGD co-applied with strychnine (black dots); area= 47.7, mean of axonal diameter= 0.308, σ2=0.003). Scale bar= 2 µm.

A $GABA_A$-R block preserves the normal structures of astrocytes during exposure to OGD-induced injury. The astrocyte viability score post-OGD was restored post-OGD in the presence of 100µM picrotoxin (mean= 1.7±0.20; n=30/13 sections; P<0.0001 vs OGD) *(see Fig6).*

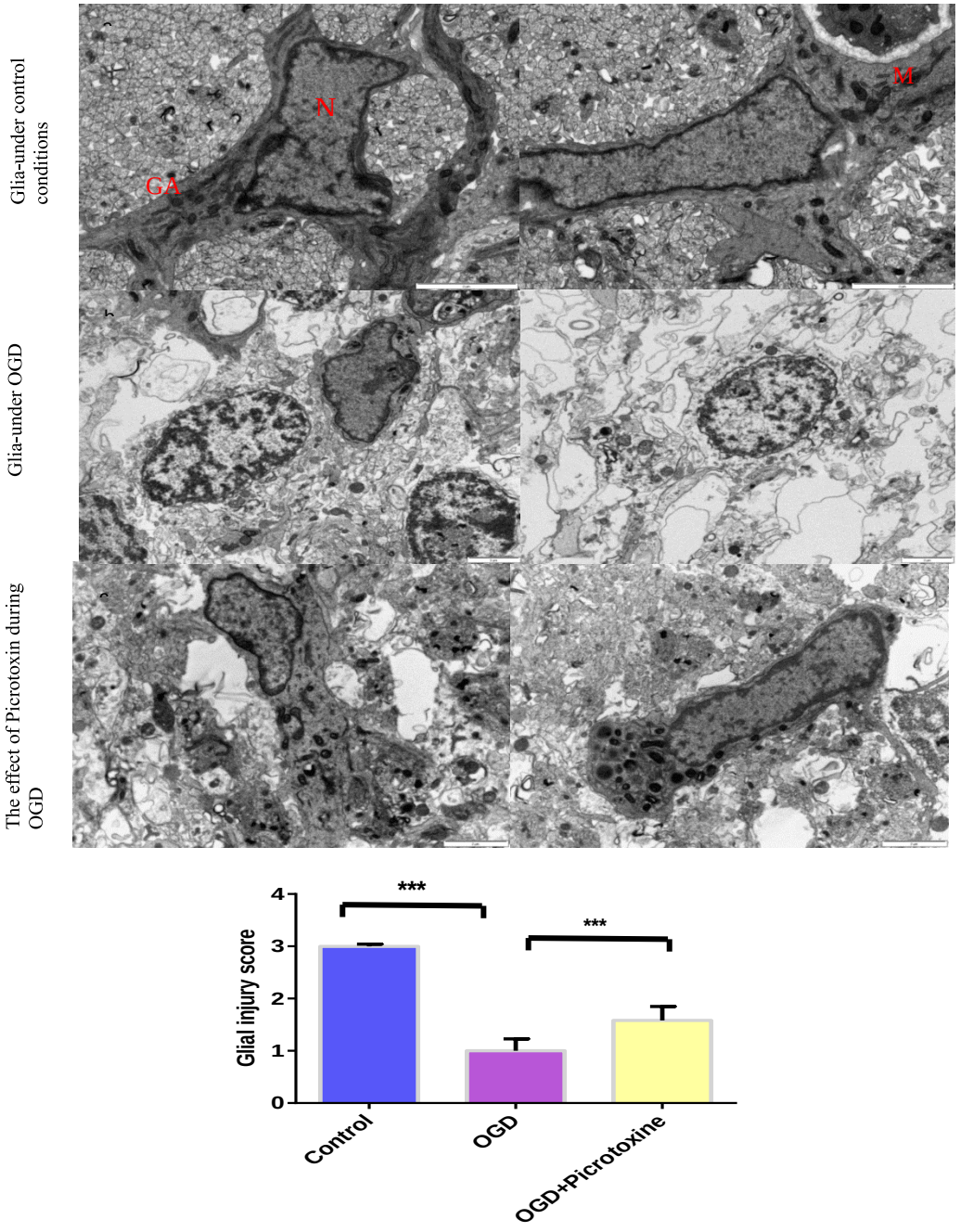



**Figure-6: GABA-R block picrotoxin (100µM) is protective against OGD-induced glial injury in P0-RONs.**
**a,** Representative normal glia from ultra-micrographs of glia during control. **b,** OGD (90 min)-induced glial injury. **c,** Representative ultra-micrographs from OGD + picrotoxin (90 min). **d,** Picrotoxin produced significant of protection against OGD-induced glial injury; ***P< 0.0001. Error bars represent SEM. Scale bar= 2 µm. N (Nucleous), M (Mitochondria) and GA (Golgi apparatus).

**Discussion**

Although the excitatory amino acids are thought to play an important role in the pathogenesis of brain ischaemia, less attention has been paid to the role of the inhibitory amino acids (Hutchinson *et al.,* 2002). Several experimental studies have shown elevated levels of excitatory amino acids accompanied by an increase in the concentration of inhibitory amino acids such as GABA and glycine in the aCSF of patients with head injuries (Palmer *et al.,* 1994), and in the extracellular space during brain ischaemia by using micro-dialysis measurements (Shimada *et al.,* 1993; Richards *et al.,* 1993). The concentration of the inhibitory neurotransmitter GABA during normal conditions is <0.002 μM - 0.02 μM, and increases during ischaemic conditions in correlation with an increase in the concentration of excitatory neurotransmitters; this indicates that brain ischaemia is associated with an increase in both inhibitory and excitatory amino acids (Hutchinson *et al.,* 2002). The increase in GABA concentration might be due to one of two mechanisms: firstly an increase in glutamate that leads to neuronal damage and consequently to an elevation in GABA, which plays a role in the pathogenesis of ischaemic injury; or secondly, the increased production and release of GABA in an attempt to counteract the excitatory amino acids as an endogenous protective mechanism (Westerink and DeVries, 1989). The injury capacity of a standard 90-min period of GABA blockade during OGD and normal aCSF upon P0-RONs was tested. During 90 min OGD, co-application of the $GABA_A$-R antagonist picrotoxin produced a decline in the CAP by the end of OGD with significant recovery after restoration of normal conditions. The data showed that picrotoxin is highly protective against OGD-induced injury in non-myelinated optic nerves. During normal aCSF, perfusion with picrotoxin resulted in a CAP increase during the first 20-30 min, and then a decline to the end of the experiment after re-institution of normal conditions. Application of picrotoxin during normal aCSF without removal of picrotoxin from the perfusate led to a gradual increase in the CAP at the end of the experiment. These results showed that $GABA_A$-R antagonist both protected the non-myelinated optic nerves from OGD-induced injury and increased CAP amplitude under normoxic conditions, indicating a protective role of $GABA_A$-R antagonist against ischaemia in developing brain. The presence of endogenous GABA in the neonatal optic nerves has been reported by several studies (Lake, 1992; Ochi *et al.,* 1993; Sakatani *et al.,* 1992); and the activation of $GABA_A$-Rs produces a partial block in the neonatal optic nerve (Sakatani *et al.,* 1991; Sakatani *et al.,* 1992), which is caused by extracellular $K^+$ accumulation and axonal depolarisation (Sakatani *et al.,* 1994; Simmonds, 1983). It is clear therefore that GABA receptors participate in brain injury at this critical period. The role of these receptors during ischaemia in neonatal brain

was investigated electrophysiologically in P0-RONs. Correspondingly; their role has been confirmed by using ultra-micrographic cross-sections in P0-RONs. The ability of blockade of these receptors to protect the neonatal RONs from OGD-induced injury at this age group has not been shown before. It was found that the number of identifiable axons following exposure to 90 min OGD was significantly restored by $GABA_A$ receptor antagonists (picrotoxin). $GABA_A$ receptor antagonist produced significant preservation of the normal structures of the astrocytes of P0-RONs following exposure to OGD-induced cellular damage. However, $GABA_A$-receptor activation evoked depolarisation in neonatal astrocytes and cells of the oligodendroglial line due to the opening of a chloride conductance (Constantinou and Fern, 2009; Sakatani *et al.,* 1991; Sakatani *et al.,* 1992; Sakatani *et al.,* 1994). No study has yet revealed that $GABA_A$-Rs elevate intracellular $Ca^{2+}$ in glia (Constantinou and Fern, 2009). Results presented here regarding the protective effect of $GABA_A$-R antagonists against OGD-induced axonal and glial injury in P0-RONs using ultra-micrograph study is consistent with electrophysiological data presented here showing protective effects against OGD-induced axonal injury in P0-RONs. It produced a mild decline in the CAPs in a reversible fashion post-OGD. The results of both electrophysiology and microscopy are consistent with a potential role of $GABA_A$-mediated injury in non-myelinated central white matter.

*Is it an indication that the axons at early point of development can release the neurotransmitter GABA during OGD even if there are no synapses? If so, where is the location of this neurotransmitter in the axons? In addition, because these axons are generally found in clusters embedded within few astrocyte processes, are these glial processes considered the source of releasing the neurotransmitters during injury and lead to stimulation of their receptors located on the axons?*

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