From muscle research to clinical applications: Do glutamate antagonists aid muscle recovery?

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Summary

It has been shown in the rat, that during the first five postnatal days, motoneurons are particularly vulnerable to excitotoxic cell death and glutamate receptors play a significant role in this time-dependent process. Various categories of glutamate blockers (MK-801, Mg, PNQX, DAP-5) have various actions on the respective receptors. Furthermore, the different response between mature and immature motoneurons following injury is attributed to the quantity of glutamate receptors on the cell membrane. The effect of these substances on the recovery of fast and slow muscles after sciatic nerve crush, at critical developmental stages, shows a variable but impressive reversal of the devastating effects on rat muscle properties, which is different between fast and slow muscles. In addition, blocking of NMDA receptors by various substances rescues motoneurons and increases the number of motor units surviving into adulthood.
In this way, glutamate receptor blockers may represent a promising therapeutic approach to retain nerve and muscle function during neurodegenerative events.

Key words: excitotoxicity, glutamate antagonists, muscle plasticity, reinnervation, NMDA, AMPA.

Historical background

Several hypotheses regarding the mechanisms underlying injury and recovery of motor function after peripheral nerve trauma have been intended for quite a long time, since the consequences of such an injury are devastating for the locomotor activity. These mechanisms undoubtedly involve resolution of the pathophysiologic events associated with this phenomenon.

The long term interactions between motor nerves and skeletal muscles are given; muscles adjust their properties to the functional requirements of their motoneurons. This interaction is mediated either by growth factors or is based on target-deprivation theory. Following denervation, nerves suffer massive loss of motoneurons and muscles undergo a broad spectrum of changes caused by the loss of normal neural activity. This type of neuronal death is attributed to the toxic effects of glutamate (overactivation of the ionotropic glutamate receptors- glutamate excitotoxicity) and indicates that such sensitivity may be involved in the long term death of these neurons. The level of excitation of axotomized motoneurons is an important factor in regulating motoneuron death [1, 2]. On reinnervation, the survived motoneurons make contact to their targets, the muscles, which are already specialized cells, and functional recovery can rarely be complete.
As it has been shown by previous studies [3, 4], motoneurons are particularly vulnerable to excitotoxic cell death during the first five days of postnatal life. Overactivation of glutamate receptors (glutamate excitotoxicity) plays a significant role in this time-dependent process [5]. In the postnatal rat, motoneurons destined to die can be rescued by agents that reduce their excitability [6-8]. Changes in glutamate neurotransmitter system play a role in functional recovery, and have been also implicated in behavioral deficits following denervation.

Glutamate system

Glutamate is one of the major endogenous CNS acids and neurotransmitters. Its receptors are known to play an important role in a series of major physiological and pathological procedures such as synaptogenesis, learning and memory, neurodegeneration (Alzheimer's disease, Parkinson's disease, epilepsy etc.).
Glutamate receptors can be divided into 2 categories based on their pharmacological and physiological properties:
1. Ionotropic receptors, named after their agonists, N-methyl-D-Asparate (NMDA), a-amino-3-hydroxy-5-methyl-4-isoxazolopropionic acid (AMPA) and Kainate acid.
2. Metabotropic receptors connected with intracellular messengers.

Ionotropic receptors of glutamate (NMDA and AMPA/kainate) have been identified throughout the brain and spinal cord and their activation leads to Ca^{2+} influx into the cell and subsequent activation of a cell death cascade[9].Glutamate can act as a widespread cytokine that can affect cell function inside and outside CNS.
Various categories of glutamate blockers (MK801, Mg, PNQX, DAP-5, etc.) have various actions on the receptors; besides, the different response between mature and imma-

ture motoneurons following injury is attributed to the quantity of glutamate receptors on the cell membrane [10]. In the present review we will present the results of blocking glutamate ionotropic receptors by substances, representative of two categories of antagonists in order to improve muscle function after axotomy.

Magnesium is a non-competitive, voltage dependent, NMDA-receptor antagonist, acting by coupling with the specific Mg2+ site within the pore of the ion channel [(11)]. Its similarity of action compared to **MK-801** has been shown in two experimental models of neuropathic pain[12]:

MK-801 [(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d] cyclohepten-5,10-imine maleate)] (dizocilpine maleate) is a non-competitive NMDA antagonist. MK-801 binds inside the ion channel of the receptor at the PCP binding site and thus prevents the flow of ions, including an influx of calcium (Ca^{+2}), through the channel[13]. The drug acts as a potent anticonvulsant, but it is not used clinically for this purpose, because it was implicated for a type of brain lesion called Olney's lesions (electron micrograph revealed that neurons in the posterior cingulate and retrosplenial cortices presented an apparent lytic breakdown of mitochondria in the large vacuoles which had become apparent 2 hours after administration of an NMDA antagonist)[14]. MK-801 is also associated with a number of negative side effects, including cognitive disruption and psychotic-spectrum reactions. It also inhibited the induction of long term potentiation. For these reasons, MK-801 is used extensively in creating animal models of schizophrenia[15].
PNQX (1,4,7,8,9,10-hexahydro-9-methyl-6-nitropyrido [3,4-f] quinoxaline-2,3-dione) synthesized in 1995, is an AMPA/kainate antagonist [16]. This compound has been also shown to antagonize NMDA receptors, acting at the glycine-binding site[16-18].
DAP-5 (D-2-Amino-5-phosphonopentanoic acid) is a selective NMDA receptor antagonist that competitively inhibits the ligand (glutamate) binding site of NMDA receptors. DAP-5 is generally very fast acting as indicated by *in vitro* preparations, and can block NMDA receptor action at a reasonably small concentration[19].

Comparison of muscle recovery after nerve injury and administration of 3 types of glutamate antagonists

We compare the time course of the functional alterations in fast and slow muscles following sciatic nerve crush on the 2nd postnatal day and the possible neuroprotective effect of Mg^{2+} [7], PNQX [8], and DAP-5 [20], administered *in vivo* daily for 2 weeks, at critical developmental stages. We also correlate our findings with the results of other researchers [21, 22] using the same experimental setting.
The animals were examined electrophysiologically for the contractile properties of extensor digitorum longus (EDL) and soleus muscles at P14, P21, P28 and adulthood (older than 2 months). Time to Peak (TTP) and Half Relaxation Time (HRT) of the Single Twitch recording was measured. Tetanic contractions were then elicited by stimulating the sciatic nerve at 10, 20, 40, 80 and 100 Hz. The fatiguability of the muscles was tested by stimulating them at 40 Hz for 250 msec every second for 3 minutes.
In addition, we studied the kinetic behavior of the animals after DAP-5 administration. 3 kinds of tests were performed at the same developmental stages.
1. The Rotarod test in which a rodent was placed on a rotating treadmill and the speed of rotation was gradually increased. The animal's ability to remain on the rotating rod was recorded.
2. Bridging: rats were placed in three different (1, 3 and 5 cm wide) narrow wooden lanes of one meter long. Two parameters were examined; the number of errors in passing the bridge and the gait type measured using a particular scale.
3. Footprint analysis: the footprint analysis was performed according to Dijkstra et al. and Klein et al. [23, 24] to evaluate hindlimb walking patterns. Briefly, the rats had to walk on strips of paper through a walk away and their hindpaws were dipped in blue fountain pen ink. The parameters examined were: stride length (distance between left and right footprints), limb rotation (angle between a virtual line through the third digit and the centre of the palm and a virtual line parallel to the walking direction) and distance between feet (distance between feet of the left and right stepping cycle).

Non parametric tests (Mann – Whitney for two independent variables and Kruskal – Wallis for more than two independent variables) were used in order to compare data, of different groups. The results are depicted in Table 1.

Muscle weight: body weight did not differ between the experimental groups. The weight in axotomized muscles was definitely reduced compared to controls. This reduction was already apparent by P14 in EDL, whereas in soleus it was evident after P28. It is also noticeable that there was a marked reduction in muscle weight from P28 to adulthood, both in the EDL and the soleus. Magnesium administration resulted in a significant muscle weight increase. In soleus, values were almost equal to those of control muscles, whereas in EDL the weight gain was less impressive. Treatment with PNQX led to a distinct higher and almost stable muscle weight ratio throughout the investigated developmental stages. The recovery of the muscle weight was greater than that achieved by magnesium administration. DAP-5 recovery was even greater for EDL (89%), but unexpectedly less for soleus (63%).

Tension development: normal muscle tension recordings were not affected by the administration of either of the above agents. As it was expected, axotomy severely affected tension development. In adult rats, single twitch of EDL was 4-9% and that of soleus 3-17% of the control side. In the same group of animals, maximal tetanic tension (developed by stimulation at 100 Hz) in EDL was only 3-6% and in soleus 13% of the control side. This marked reduction of tension developing ability of both EDL and soleus, in adult rats, is established after the first month of life. An obvious explanation is the excessive muscle atrophy that occurs gradually as the animal grows up. After treatment with Mg^{+2}, single twitch of EDL attained approximately 16% of control

and soleus improved even more, rising up to 87%. PNQX caused a more dramatic increase in EDL, up to 56% and a comparable (85%) improvement in soleus. DAP-5 administration was even more beneficial, resulting in 86% improvement in EDL and 88% in soleus.

Contraction velocity: in adult rats, EDL is normally a fast contracting muscle, whereas soleus is a slow one. Immature muscles (P14), however, are not yet differentiated into fast- or slow-contracting. Axotomy gradually converts EDL into a slow-contracting muscle. The time course of soleus contraction, on the other hand, was not altered by axotomy and the muscle remained slow-contracting in all developmental stages, in all experimental groups.
Administration of either Mg^{+2} or PNQX or DAP-5 caused axotomized EDL to regain its fast-contracting profile, up to the level to control muscles, whereas soleus time course of contractility was not affected.

Fatigue index: in adult rats, soleus is a fatigue resistant muscle, whereas EDL is not. Both immature EDL and soleus muscle, however, are fatigable and soleus gradually becomes fatigue resistant during normal development. Axotomy causes EDL to become fatigue resistant and renders soleus less fatigue resistant in adult rats. Following the administration of any of the three agents, the development of soleus into a fatigue resistant muscle is not hindered and the conversion of EDL into a fast phenotype does not take place. PNQX and DAP5 restored the profile of EDL to a greater extent than magnesium.

Movement behavior: the injection of this substance alone had no impact on animal behavior. Among the various experimental groups, crush animals had definitely lower motor scores than the controls ($p<0.05$). These differences remained throughout all ages, apart from adults, in which the gaits exhibited no significant changes. DAP5 administration in axotomized animals improved motor behaviour ($p<0.05$ compared to axotomized). For limb rotation, stride length and DBF, the difference became evident after P28.

	Mg (7)	PNQX (8)	DAP-5 (20)
Single twitch after axotomy	4.63%±0,78% EDL 16.80%±3.03% Soleus	4.63%±0,78% EDL 16.80%±3.03% Soleus	8.78% EDL 3.39%Soleus
Single twitch after treatment(% op/con)	16.59%±2.55% EDL 87.34%±21.06% Soleus	55.9±9.6% EDL 84.78±4.72% Soleus	85.81% EDL 87.22% Soleus
Maximal tetanic tension after axotomy(% op/con)	3.31%±0.30% EDL 12.44%±0.97% Soleus	3.31%±0.30% EDL 12.44%±0.97% Soleus	6.22% EDL 12.80% Soleus
Maximal tetanic tension after treatment(% op/con)	15.16%±0.89% EDL 97.00%±11.33% Soleus	58.3±4.2% EDL 87.82±11.52% Soleus	82.21% EDL 89.86% Soleus
Muscle weight after axotomy(% op/con)	10.60%±2.62% EDL 14.59%± 1% Soleus	10.60%±2.62% EDL 14.59%± 1% Soleus	11.56% EDL± 18.60% Soleus
Muscle weight after treatment(% op/con)	38.88%±5.25% EDL 90.89%± 11% Soleus	62.9±9.5% EDL 84.51±1.31% Soleus	89.01% EDL 62.79% Soleus
Time-to-peak after axotomy	77±7.89ms EDL 58±5.99ms Soleus	32±2.94 ms EDL 58±5.99ms Soleus	78.60±7.40ms EDL 54.20±3.19ms Soleus
Time-to-peak after treatment	38±7.53ms EDL 61±2.00ms Soleus	28±0.82ms EDL 61±2.00ms Soleus	43.80±6,14ms EDL 53.40±2.70ms Soleus
Half-relaxation-time after axotomy	71±11.50ms EDL 61±7.23ms Soleus	27±5.75ms EDL 61±7.23ms Soleus	71.20±5.45ms EDL 60.20±3,42ms Soleus
Half-relaxation-time after treatment	43±4.13ms EDL 60±3.77ms Soleus	24±4.00ms EDL 60±3.77ms Soleus	33.60±6,02ms EDL 68.00±2.45ms Soleus
Fatigue index after axotomy	15.6% EDL (Con:55%) 34.7% Soleus(Con:17.8%)	15.6% EDL (Con:55%) 34.7% Soleus(Con:17.8%)	17.8% EDL (Con: 48%) 34% Soleus (Con: 20.4%)
Fatigue index after treatment	9.9% EDL (Con:55%) 19.8% Soleus (Con:17.8%)	45% EDL (Con: 65%) 21% Soleus (Con: 20%)	48.2% EDL (Con:48%) 24.2% Soleus (Con: 20.4%)

Table 1. Effects of glutamate antagonists on muscle recovery after nerve damage: Comparison of the variables of muscle contraction in different experimental protocols.

Motor units

In adult animals, target deprivation of motoneurons from muscles, causes only temporary changes in their structure and function, without resulting in significant motoneuron death. During early development (first postnatal week), however, injury of a peripheral nerve, causes massive loss of motoneurons. This is reflected in the limb muscles as a reduction in the number of motor units (MU) and alterations in the muscle properties. A possible mechanism involved in trauma-induced neuronal death is thought to be excessive activation of ionotropic glutamate receptors and subsequent activation of proteases, lipases and other lytic enzymes leading to cell lysis, a process being called glutamate 'excitotoxicity'.

The number of motor units was estimated by the stepwise increments of tension, created by stimuli of different intensity. The results are presented in detail in Table 2. Our findings in muscles are consistent with results of previous work by other authors [25], as well as by our previous experience[4]: the control EDL muscle contains approximately 40 motor units, whereas soleus of 30 motor units, independently of animal age. Treatment with magnesium, MK-801 or PNQX alone did not affect the number of motor units of control muscles. In all studies, axotomy on P2 resulted in statistically significant motor unit loss in both EDL and soleus in all age groups. Motor unit loss was approx. 80% in EDL and 50% in soleus and it was already established by P14. Treatment with all agents resulted in an increased rate of survival of motor units compared to non-treated axotomized rats and, moreover, this neuroprotective effect was immediately evident, whereas in axotomized rats, MU were gradually lost until adulthood. The differences noted between magnesium or PNQX and MK-801 may in part be ascribed to the difference of the crush site (proximal plus distal crush in MK-801 treated animals). If only the animals with distal crush are considered, the values are comparable, with a clear superiority of PNQX. It is also obvious that the neuroprotective effect is already established at P14 immediately after the period of treatment.

	Mg (7)	MK-801 (21,22)	PNQX (8)
Motor units after axotomy (% op/con)	18.80%±2.93% EDL 49.87%±6.73% Soleus	13.75%±1.7% EDL 13.9%±4,1% Soleus	18.80%±2.93% EDL 49.87%±6.73% Soleus
Motor units after treatment (% op/con)	38.84%±3.01% EDL 80.56%±4.34% Soleus	45.5%±2.6% EDL 46.71%±5.2% Soleus	65.00±3.5% EDL 83.39±4.99% Soleus

Table 2. Number of motor units in reinnervated muscles after treatment with glutamate antagonists, expressed as % of the control, in various experimental protocols.

Glutamate receptor antagonists and muscle recovery after nerve injury

The results of these experimental studies show consistently, that the function of the lower extremity muscles is severely impaired following axotomy. Slow contracting muscles, like soleus, seem to be affected to a lesser extent, compared to the fast-contracting EDL. Not only did axotomy result in reduced muscle weight and strength (measured as single twitch or tetanic contraction force), but it also turned the "axotomized" EDL into a slow contracting muscle, with low fatigability. This is probably because the "fast-contracting" motor units were more susceptible to the injury and target deprivation, and therefore were necrotized after axotomy.

When glutamate antagonists (Mg, MK-801, DAP-5, PNQX) were administered to the experimental animals, in order to act as inhibitors of afferent nerve degeneration, muscle recovery improved. The improvement was better in the "slow-contracting" soleus, which achieved almost normal weight and strength (80-87% compared to the contralateral side), than in the fast-contracting EDL, which, additionally regained its "normal" properties, as a "fast-contracting" muscle.

A last point to underline is the effect of nerve injury and recovery in the number of motor units. This reflects the "surviving" motoneurons, of which the regenerate nerve consists, after injury. Fast contracting muscles (EDL) seem to have been affected more, by far, compared to slow contracting ones (soleus).Subsequently, it seems of no surprise that more slow-contracting motor units have been "salvaged" by the administration of the several "neuroprotective agents".

We have to highlight that the above experiments were based on a model of sciatic nerve axotomy in the neonatal rat. The high biological potency of the growing animal may represent a favorable situation, accelerating the injured nerve healing response. It has also to be taken into account that the administered substances may have systemic effects on the animal, reducing its overall anabolic activity, but also its mobility, thus influencing the results. That could explain, some of the discrepancies in the obtained results, among different substances used. Nevertheless, muscle atrophy and recovery, after nerve injury, is multifactorial.

These results may offer a hypothesis for further research. We are far from applying similar therapeutic interventions in clinical practice, as the exact mechanisms through which these processes take place are not yet fully understood. It is certain that nerve - muscle interactions occur in both directions. The damaged nerve affects the muscle, the absence of a target - muscle affects the nerve.

Glutamate receptors antagonists. Is there a role in clinical practice?

Glutamate induced excitoxicity has been linked to both acute (ischemia, trauma) and chronic (epilepsy, Alzheimer's disease, amyotrophic lateral sclerosis, multiple sclerosis, Parkinson's disease, schizophrenia, neuropathic pain) conditions affecting the nervous system[26]. Both human and animal studies have demonstrated that glutamate receptor activity can also be modulated by use of substances. Could inhibition of excitotoxicity, mediated by glutamate, acting as a neurotransmitter, reverse the effects of neuronal tissue damage?

The aim of experimental studies was to investigate the

pathophysiological pathways associated with neuronal loss mediated by glutamate (neurotransmission). Several in vitro and in vivo models have been developed and various pharmaceutical substances (antagonists of glutamate receptors) have been applied.

Acute conditions affecting the central nervous system (CNS), such as stroke and brain trauma, have traditionally been the focus of research on excitotoxicity. Indications of neuroprotection in animal models rose hope that humans could benefit as well. Human trials, however, failed to show that glutamate receptor antagonists offer neuroprotection in patients suffering a stroke[27] or brain trauma[28]. Despite extensive research, no pharmacological modalities have been found effective regarding neuroprotection after acute neuronal damage. In fact, it seems that glutamate receptors are hiding their secrets, and scientist should go back one step, and try to investigate their role in more detail. In contrary, clinical results regarding chronic conditions like Alzheimer disease, epilepsy etc. are more promising[29, 30], as substances like memantine (AMPA receptor antagonist) have been proved to be beneficial[31].

In order to rationalize these results, a hypothesis suggested that, although glutamate is related to excitotoxicity resulting in neuronal cell death in the acute phase, its "usual" physiological role, which includes promotion of neuronal function and survival, is resumed at a later stage[27]. If this is true, glutamate receptor antagonists may have short term beneficial action after acute nerve damage, but will hinder neuronal survival later.

We have to note, however, that in several cases, drug effect may be multifactorial and so the isolated study of one class of receptors is not feasible. For example, riluzole, the only therapeutic agent available for amyotrophic lateral sclerosis (ALS), preferentially blocks TTX-sensitive sodium channels, which reduces influx of calcium ions and indirectly prevents stimulation of glutamate receptors. However, the action of riluzole on glutamate receptors has been controversial, as no binding of the molecule has been shown on any known receptor. It seems that its potent glutamate uptake activator activity is responsible for many of its effects[32].

During the last decade, our team has investigated the effect of glutamate receptor antagonists on motoneuron survival, in animal models of Wistar-Albino rats, after peripheral nerve injuries, as described earlier[7, 8, 33]. We found that inhibition of glutamate-mediated neurotransmission in immature rats that sustained peripheral nerve injury resulted in increased motoneuronal survival and had a positive effect on hindlimb muscle properties. Our results were indicative that there is a potential role for pharmacological interventions in acute peripheral nerve trauma (axotomy) in humans. No human clinical studies are available though and we believe we are far from seeing glutamate receptor antagonists being used in everyday clinical practice. These pharmaceutical agents often very toxic[34] and their pathophysiological role remains still not completely understood. Furthermore, the failure of recent clinical trials to demonstrate neuroprotective action after administration of NMDA-antagonists in patients with stroke, induced skepticism rather than hope.

Conclusion

Substantial evidence has been provided by neurophysiologic, neuroanatomical, and molecular biology studies in animals, that the adult neuromuscular system is capable of significant functional plasticity.

The level of excitation of axotomized motoneurons is an important factor in regulating motoneuron death. During this period of arrested muscle development, the spinal cord circuitry continues to develop and motoneuron activity assumes a more mature pattern, i.e. they fire more often and at higher frequencies[35]. On reinnervation by axons of these motoneurones, many muscle fibres that had their development arrested, are unable to withstand this new pattern of activity and die[36, 37].

It is becoming obvious that the survival of both motoneuron and muscle depends on the accurate timing of changes of their molecular composition that allow them to carry out the functions required[38]. The theoretical framework for understanding recovery of function is still evolving. Glutamate is the major neurotransmitter in the CNS and any synaptic modifications that may be necessary for functional reorganization may be mediated by NMDA receptors. Down regulation of these receptors may be an adaptive response to injury, and may, at least in rats, favor recovery.

The clinical implication, which is corroborated by the findings in experimental animals, is that following a traumatic injury to the motor nerve, the pharmacological inhibition of neural activity may lead to functional improvement of locomotion.

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