CHARACTERIZATION OF EXCITATORY AMINO ACID NEUROTRANSMITTERS
AT MOTONEURON SYNAPSES CONTACTING RENSHAW CELLS

A dissertation submitted in partial fulfillment of the
requirements of the degree
of Doctor of Philosophy

By

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ABSTRACT


Motoneurons are among the best studied neurons in the central nervous system. The motoneuron synapses have been well characterized in the periphery where they release acetylcholine at the neuromuscular junction. However excitatory amino acids also seem to be released from motoneuron terminals in the periphery, and centrally at their synapses contacting Renshaw cells. Although excitatory amino acids are suggested to be released from motoneuron synapses it is not known which excitatory amino acids (either aspartate or glutamate) are released, nor is the mechanism for their release known.

To examine the presence and mechanism of release for aspartate and glutamate at motoneuron synapses on Renshaw cells, several immunocytochemistry experiments using both epifluorescence and electron microscopy techniques were used to determine if any of the known vesicular glutamate transporters (VGLUTs) or other transporters were present and to quantify the enrichment of aspartate and glutamate in these terminals. Moreover, immunofluorescent experiments using the Hb9::EGFP mouse model were done to confirm the specificity of VACHT immunolabeling for identifying motoneuron contacts on calbindin immunoreactive (-IR) Renshaw cells.

The results from these experiments show that the known VGLUTs are not detectable at motoneuron contacts on Renshaw cells, and therefore aspartate and glutamate must be released via a VGLUT-independent mechanism. Immunofluorescent experiments with HB9::EGFP mice confirmed that VACHT is an appropriate marker for labeling motoneuron contacts on Renshaw cells. Electron microscopy experiments
determined that both glutamate and aspartate are enriched in VACHT-IR contacts on Renshaw cells. Further immunofluorescent experiments looking for potential transporters for packaging excitatory amino acids into synaptic vesicles revealed that both SLC10A4 and SLC17A5 (members of the solute carrier protein family that also include the VGLUTs) were present in motoneurons. SLC10A4-IR was present in motoneuron terminals, whereas SLC17A5-IR was restricted to the motoneuron somata.

In conclusion, aspartate and glutamate are enriched in motoneuron terminals contacting Renshaw cells. However, these synapses lack detectable VGLUT-IR. This suggests that there is another mechanism present for packaging aspartate and glutamate into vesicles. Future studies examining SLC10A4 are needed to determine if it is capable of transporting aspartate and glutamate into synaptic vesicles for release at motoneuron synapses.
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I. Introduction

Motoneurons are among the best studied neurons in the central nervous system (CNS). Their peripheral projections to the neuromuscular junction (NMJ) have been well characterized and the release of acetylcholine at this synapse is well known. Acetylcholine was one of the first neurotransmitter identified thanks to the accessibility of the NMJ connection (Dale et al. 1936). However, other data suggested that additional neurotransmitters might be released from motoneurons. Using electron microscopy high levels of glutamate were observed inside motor axon end-plates at NMJs (Waerhaug and Ottersen, 1993) and motoneuron released glutamate was shown to presynaptically modulate NMJ function (Malomouzh et al., 2003; Pinard et al., 2003). The prevailing thought for many years was that nicotinic acetylcholine receptors were the only postsynaptic receptors expressed in muscles and this might have slowed the search for other neurotransmitters that could be released from motor axons. New data however suggest that AMPA and NMDA receptors are located postsynaptically at NMJs (Mays et al., 2009), but the reasons why they are not responsive to glutamate proposed to be released from the motor end plate are unknown. Interestingly, muscle fibers connected to spinal cord white matter tracts through a peripheral nerve graft become innervated by collaterals of descending glutamatergic axons that effectively activate postsynaptic AMPA and NMDA receptors in muscle (Brunelli et al., 2005).

Motoneuron axons also have recurrent collaterals inside the spinal cord and these collaterals make synapses with Renshaw cells, an interneuron that provides feedback inhibition to the same motoneurons (Renshaw, 1946; Eccles et al., 1954). The motor axon synapse on Renshaw cells was initially characterized as cholinergic (Eccles et al., 1954; 1961). In fact the release of acetylcholine in both the peripheral and central
branches of motor axons was the observation that lent support to Dale’s Principle: “A neuron releases the same transmitter substance from all its synaptic terminals” (Dale, 1935). Two incorrect interpretations of Dale’s principle became somewhat widespread. First is that the actions of presynaptic neurons should be the same in all postsynaptic targets. Second, that neurons release only one neurotransmitter. However it is now well known that the exact postsynaptic action depends on the type of postsynaptic receptors present. Moreover most neurons co-release more than one neurotransmitter substance. The peripheral presence and release of excitatory amino acids from the peripheral terminals of motor axons at the NMJ suggests that they would also be released centrally. This possibility receives strong support from recent pharmacological studies of the motor axon-Renshaw cell synapse (Mentis et al., 2005; Nishimaru et al., 2005; Lamotte d’Incamps & Ascher, 2008), as well as from earlier works.

In early in vivo extracellular recordings of cat Renshaw cells, acetylcholine receptor antagonists (nicotinic and muscarinic) were unable to completely inhibit the firing activity of Renshaw cells evoked after ventral root motoneuron axon stimulation (Eccles, et al. 1954). Furthermore, after stimulating ventral roots and recording intracellularly from rat motoneurons, the disynaptic recurrent inhibition was not completely blocked by acetylcholine receptor antagonists (Schneider & Fyffe, 1992). Whole-cell voltage clamp recordings of Renshaw cells also showed that synaptic currents evoked by ventral root stimulation could not be completely inhibited by acetylcholine receptor antagonists (Dourado & Sargent, 2002). These studies indicated that it is highly likely that there is more than acetylcholine being released at motoneuron synapses on Renshaw cells. More recently, two parallel studies using whole-cell patch clamp of
Renshaw cells following ventral root stimulation in the neonatal mouse spinal cord showed that in the presence of acetylcholine receptor antagonists neither excitatory postsynaptic potentials (EPSPs) nor excitatory postsynaptic currents (EPSCs) could be completely blocked. However, the addition of NMDA and AMPA/kainate receptor antagonists removed the remaining EPSPs and EPSCs (Mentis et al., 2005; Nishimaru et al., 2005). Furthermore, nicotinic receptor antagonists did not completely inhibit Renshaw cell firing activity in response to motor axon excitation; the addition of glutamatergic receptor antagonists was necessary to block all activity (Lamotte d’Incamps & Ascher, 2008). All together, this evidence suggests that excitatory amino acids (EAA; i.e., glutamate and/or aspartate) could be co-released with acetylcholine from the central motoneuron synapses on Renshaw cells. Moreover, Renshaw cells express postsynaptic receptors capable of responding to EAA neurotransmitters co-released with acetylcholine from motor axons (Mentis et al., 2005; Nishimaru et al., 2005; Lamotte d’Incamps & Ascher, 2008).

The objective of the work that will be presented in this dissertation is to investigate, using immunocytochemical techniques, whether glutamate or aspartate and their release machinery are expressed by motoneuron synapses on Renshaw cells. Briefly excitatory amino acid co-release capable of activating AMPA/NMDA receptors on Renshaw cells might help us better understand: 1) the time course of the motor axon EPSPs evoked on Renshaw cells (very long as compared to the short time course of muscle end-plate potentials EPPs); 2) the possibility that NMDA receptor activity is implicated in the maturation of motor axon synapses on Renshaw cells; 3) the possibility of non-cholinergic motoneuron actions in the spinal cord.
II. Background

Renshaw cell physiology

The introduction of a class of spinal interneurons, namely Renshaw cells, began from electrophysiological recordings in the cat spinal cord in vivo preparation showing that local motoneurons could be activated or inhibited by antidromic volleys evoked by stimulating ventral roots. It was postulated that an interneuron was responsible for the inhibition of the motoneurons, as local motoneurons were inhibited after a very short latency following ventral root stimulation (Renshaw, 1941). Later, a group of interneurons was found in lower lamina VII that exhibited high frequency bursting activity following antidromic stimulation of ventral roots (Renshaw, 1946). It was thereafter further confirmed that a population of interneurons or “Renshaw cells” (named for the original discoverer) indeed exhibited the characteristic high frequency bursting activity when ventral roots were stimulated, and in turn motoneurons were inhibited (Eccles, et al., 1954). This bursting activity is characterized by an initial spiking of up to 1500 Hz that slowly declines over a period of 50-100 msec (Frank & Fuortes, 1956; Curtis & Eccles, 1958). Simultaneous recordings from Renshaw cells and motoneurons show that a single spike evoked in motoneurons can evoke the well characterized burst of spiking activity in Renshaw cells (Van Keulen, 1981). Underlying this high frequency bursting response is a long excitatory postsynaptic potential (Eccles et al., 1961; Walmsley & Tracey, 1981). More recently, using whole cell current clamp recordings in neonatal rodent spinal cord slices, it was revealed that the response of Renshaw cells consist of an initial double spike followed by a later train of action potentials (Lamotte d’Incamps & Ascher, 2008). The characteristic response of Renshaw cells to motoneuron
input allows tight coupling of motoneuron activity and Renshaw cell responses and increases the duration of recurrent inhibition lasting tens of milliseconds. The exact reasons why synapses involved in recurrent inhibition are longer-lasting than, for example, reciprocal inhibition, is not well understood.

In contrast to their response to ventral root or motoneuron stimulation, Renshaw cells modulate their firing activity differently in response to other physiological inputs. For example, Renshaw cells’ firing following dorsal root stimulation is not as long as the burst initiated by ventral root stimulation (Curtis et al., 1961).

**Renshaw cell anatomy**

Anatomically Renshaw cells are located in ventral lamina VII (Thomas & Wilson, 1965). Intracellular labeling of cat Renshaw cells showed that they are a heterogeneous population of small interneurons made up of both fusiform and multipolar cell types with soma diameters between 10 and 15 µm (Jankowska & Lindström, 1971). They usually have 3-8 primary dendrites with an arborization radius of about 800 to 1,100 µm (Fyffe, 1990; Alvarez et al., 1997). Putative Renshaw cells in lower lamina VII of the monkey spinal cord were proposed to be immunoreactive to the calcium binding protein, calbindin-d28k (Arvidsson et al., 1992). However, calbindin is not present in all Renshaw cells in the cat (Carr et al., 1998) and not all calbindin-IR cells in the spinal cord are Renshaw cells. Fortunately, all Renshaw cells (in cat, rodents, monkey, human etc.) display exceptionally large gephyrin clusters that are specific to this cell type and permit their identification (Alvarez et al., 1997). Gephyrin forms the postsynaptic clustering scaffold for glycine and GABA\(_A\) receptors. Large sized clusters are related to
the presence of inhibitory synapses in Renshaw cells with exceptionally large numbers of postsynaptic receptors and very large postsynaptic inhibitory currents (Gonzalez-Forero & Alvarez, 2005). Therefore, the presence of large gephyrin clusters is a preferred marker for Renshaw cells in the cat (Alvarez et al., 1997) and also has confirmed that the ventral LVII cluster of calbindin-IR neurons in rodent (rat and mice) spinal cords are mostly (>90%) Renshaw cells in adult and during development (Carr et al., 1998; Geiman et al., 2000; Sapir et al., 2004). In this study we used calbindin as the most convenient marker for Renshaw cells in rats and mice.

The location of Renshaw cells is related to their specific input from motoneurons. Intracellular axon labeling of motoneurons in the cat determined that the terminal arbors of intraspinal motor axon recurrent collaterals are focused in ventral lamina VII where Renshaw cells are located (Cullheim et al., 1977; Cullheim & Kellerth, 1978; Lagerbäck et al., 1981; Cullheim & Ulfhake, 1985). Medium to large size cholinergic contacts, presumably from motoneurons, are observed on Renshaw cell dendrites and to a lesser extent their somas (Alvarez et al., 1999; Geiman et al., 2000). The motor axon input is located more distally than the powerful inhibitory synapses that almost fully cover the proximal dendrite and cell body membrane.

**Renshaw cell function**

At the segmental level Renshaw cells participate in recurrent inhibition. Renshaw cells inhibit motoneurons in the same motor nuclei from which they receive inputs (Eccles et al., 1961a,b). Originally, Renshaw cells were thought to only project to motoneuron pools in close proximity (Eccles, 1961). However, further research
determined that there is extensive convergence of inputs on Renshaw cells from motoneurons supplying synergistic muscles, while Renshaw cells receive little or no input from antagonists. In turn Renshaw cells synapse back onto the same motor pools they receive input from (Ryall, 1981). Thus, different pools of Renshaw cells preferentially couple to motoneurons projecting to either flexor or extensor muscles. As a result during locomotor-like rhythmic activity some Renshaw cells are active during the flexor phase and others during the extensor phase (McCrea et al., 1980; Pratt and Jordan, 1987; Nishimaru et al., 2006). Moreover, during fictive locomotion Renshaw cell activity occurs only after the motoneuron it is coupled to becomes active (McCrea et al., 1980). In the *in vivo* cat preparation, Renshaw cell firing is maximal toward the end of the extensor or flexor motor burst and slightly exceeds its duration (Pratt and Jordan, 1987). Analysis of motoneuron firing during fictive locomotion while pharmacologically blocking motoneuron-Renshaw cell synapses (Noga et al., 1987) suggested that Renshaw cells reduce motoneuron firing rates during locomotor bursts and might also contribute to the termination of motoneuron activity at the end of the cycle.

Renshaw cells also send inhibitory input to IaINs that inhibit the motoneurons of antagonistic muscles (Hultborn et al., 1971). Renshaw cell activation therefore inhibits the same motoneurons from which they receive input while simultaneously disinhibiting antagonistic motoneurons. By controlling IaIN activity Renshaw cells facilitate the activation of antagonists at the end of a cycle and can also adjust the level of co-contraction around single joints.

Yet, recurrent inhibition is not intrinsic to all motor pools. The development of techniques for intracellular labeling with horseradish peroxidase motoneuron dendrites
and axons made it possible to study the arborizations of intraspinal motor axon recurrent
collaterals and their contacts on Renshaw cells from single electrophysiologically
identified motoneurons. Using this technique in the cat, no axon collaterals projecting to
Renshaw cells were observed from motoneurons innervating the short plantar muscles of
the foot, but many were observed from motoneurons innervating more proximal muscles
(Cullheim & Kellerth, 1978a; McCurdy and Hamm, 1992). A lack of recurrent
collaterals from motoneurons projecting to more distal muscle targets, suggests a lack of
recurrent inhibition in these muscles. Functionally motor units innervating distal muscles
of hand and feet seem to receive little or no recurrent inhibition (Hamm, 1990; Katz et
al., 1993; Turkin et al., 1998). These results have been interpreted to suggest that
recurrent inhibition is more important for modulating gross movements (more proximal
muscles) than for fine motor control (more distal muscles).

As detailed as these descriptions are regarding Renshaw cell connectivity, they do
not fully reveal the exact function of recurrent inhibition in motor control or its
contribution to the maintenance of posture and movement. One model suggests that
Renshaw cells modulate the input-output gain of motoneurons (Hultborn et al., 1979 and
2004). It was therefore suggested that during weak contractions, when motoneurons are
more weakly activated, Renshaw cell function is facilitated and motoneurons are more
inhibited, however during stronger contractions Renshaw cells are partially inhibited
allowing increased firing of motoneurons in response to descending or segmental
excitatory inputs. Opposite effects were proposed to occur in antagonist motoneurons
because of Renshaw cell inhibition or disinhibition of IaINs. However this theory does
not explain why motoneurons generating stronger force (fast fatigable motor units)
generate the largest input to Renshaw cells (Cullheim and Kellerth, 1978) or the known higher amount of recurrent inhibition received by proximal antigravity extensor motoneurons compared to slower or weaker more distal motor units (reviewed above). The exact role Renshaw cells play in motor control is still a much debated topic.

**Renshaw cell synaptic pharmacology**

Renshaw cells mediate recurrent inhibition by releasing inhibitory neurotransmitters at synapses on motoneurons. Initially, Renshaw cell synapses on motoneurons were described as strictly glycineric as they were inhibited by the glycine receptor antagonist strychnine (Curtis et al., 1968, 1971). Later studies showed that Renshaw cell synapses could co-release GABA, because the GABAergic antagonist bicuculline could further inhibit recurrent IPSPs in motoneurons following the application of strychnine (Cullheim & Kellerth, 1981; Schneider & Fyffe, 1992). This observation was first interpreted as possible independent GABAergic or glycineric Renshaw cells. However, more recent studies established the co-release of both neurotransmitters at inhibitory synapses in the ventral horn of the spinal cord (Jonas et al., 1998). The co-release of GABA and glycine at the same synapse is able to activate the opening of channels associated with GABA and glycine receptors. Each type of receptor has different biophysical properties and differentially influences peak amplitude (dominated by the glycine component) and duration (determined in large part by the GABA component) of inhibitory currents (Jonas et al., 1998; Gonzalez-Forero & Alvarez, 2005). Whole-cell patch clamp recordings in abducens motoneurons demonstrated the higher efficiency of mixed glycine/GABA synapses for modulating
motoneuron firing (Russier et al., 2002). Accordingly in recurrent inhibition, the presence of both receptor types is expected to more effectively suppress motoneuron firing by increasing both the strength and duration of the IPSP.

**Pharmacology of motoneuron synapses on Renshaw cells**

Historically, the excitatory receptors participating in the recurrent inhibition loop on Renshaw cells were believed to be primarily cholinergic. Microelectrophoretic techniques showed that Renshaw cells are sensitive to nicotinic and muscarinic receptor antagonists (Curtis & Eccles, 1958; Curtis et al., 1961; Curtis & Ryall 1966a, b, c; Headley et al., 1975). In early studies nicotinic and muscarinic receptor antagonists administered intravenously both diminished Renshaw cell firing in response to ventral root stimulation (Curtis et al., 1961; Curtis & Ryall, 1966b). However, most of the Renshaw cell activation was primarily due to nicotinic receptors and the expression of muscarinic receptors seem to be species-specific as they were not detected in the rat (Headly et al., 1975).

Two populations of nicotinic receptors have recently been characterized in Renshaw cells. One population is hetero-oligomeric α4β2 subunits, the other homomeric α7 receptors (Dourado & Sargent, 2002; Lamotte d’Incamps & Ascher, 2008). The presence of the α4β2 subunit containing nicotinic receptors explains the high sensitivity of Renshaw cells to the antagonist dihydro-β-erthroidine (dHβE) (Curtis et al., 1976). dHβE is highly specific for α4 containing nicotinic receptors (Chavez-Noriega et al., 1997). Methyllycaconitine (MLA), another cholinergic receptor antagonist, is a specific α7 subunit blocker (see, Lloyd & Williams, 2000). Both α4β2 and α7 containing
nicotinic receptors in the Renshaw cell contribute to the synaptic currents evoked by motor axons. dHβE eliminates a slower component of the nicotinic current, whereas MLA eliminates a fast rising and fast decaying component (Lamotte d’Incamps & Ascher, 2008). However, it should be noted that none of the cholinergic receptor antagonists, even when given in combination, can completely inhibit Renshaw cell activation or the EPSPs/EPSCs following ventral root stimulation (Eccles et al., 1954; Schneider & Fyffe, 1992; Dourado & Sargent, 2002; Mentis et al., 2005). This led several investigators, including us, to test the possibility that activation of AMPA and NMDA receptors is involved in the generation of the motoneuron to Renshaw cell EPSP (Mentis et al., 2005; Nishimaru et al., 2005; Lamotte d’Incamps & Ascher, 2008).

Other inputs on Renshaw cells

Despite the predominant focus on the motor axon inputs that modulate Renshaw cell firing, there are other sources of inputs converging on Renshaw cells. One source of excitatory input on Renshaw cells comes from muscle proprioceptive primary afferents (Mentis et al., 2006). Monosynaptic proprioceptive inputs were believed not to exist in adult Renshaw cells (Curtis & Ryall, 1966), however recent investigations in our lab demonstrated their presence in neonatal Renshaw cells. Interestingly this input is not fully removed with maturation but “functionally de-selected” such that the density of synapses decreases with age (Mentis et al., 2006) and AMPA receptors are removed from the postsynaptic region (Zerda & Alvarez, unpublished). Both processes likely weaken this synapse such that it becomes subthreshold and has little effect on Renshaw cell firing.
modulation in adult, as found by early investigators (Renshaw, 1946; Eccles et al., 1956; Curtis & Ryall, 1966).

Another source of excitatory input on Renshaw cells are spinal cord interneurons. Renshaw cells receive many synapses that can be labeled with VGLUT2 antibodies, a marker of glutamatergic synapses from spinal interneurons (VGLUT2 or isoform 2 of the vesicular glutamate transporter, will be reviewed later). One population of ventral medial glutamatergic interneurons believed to be involved in the stabilization of motor rhythms gives excitatory inputs onto Renshaw cells (Zhang et al., 2008). It is likely that many other groups of excitatory interneurons target the Renshaw cells and in part mediate the known facilitation of Renshaw cell activity by descending motor systems. Some descending systems like the serotonergic and noradrenergic system also directly contact Renshaw cells but these inputs generally have very low densities (Carr et al., 1999; Zheng & Fyffe, unpublished). Nonetheless Renshaw cell activity is effectively modulated by serotonergic and noradrenergic agonists and antagonists (Jordan & McCrea, 1976; Gajendiran, 2006).

Finally, Renshaw cells receive many inhibitory inputs from local spinal interneurons. As mentioned above, the density of inhibitory receptors is very high on the Renshaw soma and proximal dendrites, as can be seen by the high density of very large gephyrin clusters on their membranes (Alvarez et al., 1997; Geiman et al., 2000). Gephyrin is a scaffold protein for the clustering of GABAergic and glycinergic receptors. This clustering of both inhibitory receptors leads to inhibitory currents in Renshaw cells that have a large peak amplitude fast component due to the activation of glycine receptors, and slow decay time due to GABAA receptors (Gonzalez-Forero & Alvarez,
2005). These inhibitory synapses generate a very large inhibitory current that is believed to be designed to match during postnatal development the strength and duration of motor axon excitatory inputs (Gonzalez-Forero & Alvarez, 2005). The presence of high strength inhibitory inputs might allow for better modulation of the gain of the recurrent inhibitory loop. Continuous modulation of Renshaw cell inhibitory actions on motoneuron firing is a central element of current models of Renshaw cell function (Hultborn et al., 1971). The exact interneuronal sources of inhibitory inputs on Renshaw cells remain unknown. The only known inhibitory input on Renshaw cells comes from Renshaw cell contacts on other Renshaw cells (Ryall, 1981).

Motor axon synapses

Motoneurons extend their axons into the periphery where they make contact with their muscle targets and form synapses at the NMJ. Inside the spinal cord motor axons send collaterals to Renshaw cells before exiting in the ventral root. Motoneuron terminals in the periphery release acetylcholine (Fatt & Katz, 1952). In fact, this is where this neurotransmitter was first discovered (Dale et al., 1935).

The cholinergic properties of motoneurons have been abundantly described. They express choline acetyltransferase (ChAT), an enzyme responsible for producing acetylcholine, and the vesicular acetylcholine transporter (VACHT), which packages acetylcholine into synaptic vesicles (Erickson et al., 1994). ChAT and VACHT can be immunocytochemically detected in all large neurons in LIX of the spinal cord (Barber et al., 1984; Phelps et al., 1988; Schäfer et al., 1994; Hellstrom et al., 1999). ChAT
immunolabeling is generally used to label motoneuron cell somata, while VACHT immunolabeling is highly concentrated in the cholinergic synapses.

As reviewed above, close examination of classical Renshaw cell papers and new investigations have raised the possibility of co-release of excitatory amino acids and the presence of postsynaptic AMPA and NMDA receptors. The expression of these receptors or of the molecules necessary to package these neurotransmitters into synaptic vesicles has not yet been investigated.

Another piece of evidence showing it is possible that more than acetylcholine is released from motoneurons comes from invertebrates. Glutamate is the motoneuron neurotransmitter in invertebrates, as initially discovered in Drosophila (Usherwood & Machili, 1966) and crayfish (Takeuchi & Takeuchi, 1964). High levels of glutamate were also identified at the mammalian NMJ using electron microscopy (Waerhaug and Ottersen, 1993). Further evidence for glutamate release in mammals includes the presence of glutamate receptors (AMPA and NMDA receptors) at the NMJ. Earlier studies localized NMDAR1 subunits to the neuromuscular junction, but failed to describe the subcellular localization (Berger et al., 1995; Grozdanovic & Gossrau, 1998; Luck et al., 2000). More recently AMPA and NMDARs were localized near bungarotoxin labeled nicotinic receptor fields (Mays et al., 2009). In addition, glutamate released from motoneurons is also able to activate presynaptically NMDA receptors and modulate non-quantal acetylcholine release from the NMJ (Malomouzh et al., 2003). Moreover, activation of metabotropic glutamate receptors decreased quantal-release of acetylcholine and effectively reduced end-plate potential amplitude (Pinard et al., 2003). Although all these actions are considered “glutamatergic”, the exact excitatory amino acid
neurotransmitter released from motor axons, glutamate or aspartate, has never been directly confirmed. Finally experimental muscle innervation of denervated muscle by descending spinal glutamatergic axons bridged through peripheral nerve grafts switched muscle sensitivity from cholinergic to glutamatergic and muscle fibers then express AMPA/NMDA mediated EPPs (Brunelli et al., 2005). Altogether, these data indicate the presence of excitatory amino acids in motoneuron terminals in the periphery. According to Dale’s principle they should be present at central motoneuron terminals as well.

**Glutamate and aspartate: neurotransmitters in the CNS and developmental actions**

Although it was known for many years that glutamate had excitatory effects on central neurons (Hayashi, 1954), it wasn’t until much later that it was clearly defined as a neurotransmitter (for review see Roberts et al., 1981). Excitatory amino acids in the CNS are ubiquitous and also used for many metabolic processes. Therefore determining their accumulation and release from synapses has been a difficult process. Specific fixation techniques are necessary to determine immunocytochemically if excitatory amino acids are accumulated in synaptic vesicles (Storm-Mathisen et al., 1995). Glutamate is now broadly accepted as the primary excitatory neurotransmitter in the CNS. Later, aspartate was also defined as a neurotransmitter. The excitatory action of aspartate was first determined when its application to motoneurons resulted in membrane depolarization (Curtis & Watkins, 1960), however because of its more stringent fixation requirements it has been difficult to demonstrate its accumulation at synapses. It has also been difficult to distinguish aspartate’s postsynaptic actions from released glutamate (Gundersen & Storm-Mathisen, 2000).
There are three known types of postsynaptic ionotropic glutamate receptors in the CNS: 1) α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid AMPA receptors [comprising glutamate receptor (GluR) 1–4 subunits]; 2) kainate (KA: high affinity; comprising GluR5–7 and KA1, KA2 subunits); and 3) N-methyl-D-aspartate NMDA receptors (comprising NR1, NR2A-D and NR3A subunits) (for review, see Barnard, 1997). Different subunits confer specific physiological properties to the receptors upon ligand binding. In the CNS AMPA and NMDA receptors are frequently colocalized in the postsynaptic membrane (Bekkers & Stevens, 1989). Developmentally, several events determine the maturation of glutamatergic synapses: 1) increased strength in AMPA receptor mediated transmission (Wu et al., 1996; Petralia et al., 1999) and 2) a switch in the subunit composition of NMDA receptors (NR2A for NR2B subunits) during synaptic maturation (Monyer et al., 1994; Sheng et al., 1994; Flint et al., 1997) and 3) at many synapses, particularly on motoneurons, down regulation of synaptic NMDA receptors (Arvanian et al., 2004). All these events are directly or indirectly linked to NMDA receptor activation.

Glutamate is known to bind and activate AMPA, NMDA and kainate receptors. Aspartate however, only has significant effects on NMDA receptors (Patneau & Mayer, 1990; Curras & Dingledine, 1992). NMDA receptors have three known physiological agonists (glutamate, aspartate, and glycine) and the binding of glutamate or aspartate together with glycine most efficiently opens the receptor allowing calcium to enter the cell (Johnson & Ascher, 1987; Kleckner & Dingledine, 1988). The binding site for aspartate or glutamate is in the NR2 subunit, whereas glycine binds to the NR1 subunit (Dingledine et al., 1999). The NR3 subunit is present predominantly during development
and can depress NMDA receptor function (Sucher et al., 1995). As NMDA and AMPA receptors are often colocalized the postsynaptic response frequently has two components distinguished by their time course. NMDA receptors contribute to the slow component and AMPA receptors contribute to the fast component (for example, see Mentis et al., 2005). The NMDA receptor’s most distinctive feature is its voltage-dependent Mg$^{++}$ block, such that in addition to ligand binding the receptor requires membrane depolarization for opening (Mayer et al., 1984). Differently from AMPA receptors, opening of NMDA receptors allows calcium entry into the cell and activates a large number of signaling cascades that target many intracellular mechanisms including changes in gene expression. Therefore activation of NMDA receptors triggers many plastic changes in postsynaptic neurons and their functional importance goes beyond generating the synaptic current necessary for EPSPs.

NMDA receptors are found throughout the spinal cord and have important roles in development. On motoneurons, application of the NMDA receptor antagonist MK-801 during early postnatal ages reduces the size of the dendritic arbor in motoneurons (Kalb, 1994). Moreover, the presence of NR3B in the early postnatal times is important to the formation of the dendritic arbor and over expression of NR3B leads to an increased length and complexity of the dendritic arborization in motoneurons (Prithviraj & Inglis, 2008). The contribution of glutamate receptors to developmental maturation of Renshaw cells and their synapses is unknown.

Vesicular glutamate transporters or VGLUTs.
VGLUTs package glutamate into vesicles for release at synapses. None of the VGLUT isoforms known to date are capable of transporting aspartate. Basal glutamate levels in neurons are enough for accumulation into synaptic vesicles through VGLUTs. For example, induced expression of VGLUTs in GABAergic inhibitory neurons results in synaptic GABA/glutamate co-release (Takamori et al., 2000). The first (VGLUT1) of the three known types of glutamate transporters (VGLUT1-3) was initially identified as the brain-specific Na\(^+\)-dependent inorganic phosphate transporter (BNPI) (Bellocchio et al., 2000; Takamori et al., 2000). The second transporter (VGLUT2) was identified from the differentiation-associated Na\(^+\)-dependent inorganic phosphate transporter (DNPI) (Aihara et al., 2000; Takamori et al., 2001; Herzog et al., 2001; Varoqui et al., 2002). VGLUT1 and VGLUT2 are quite similar in structure, but differ in that VGLUT1 depends only on the membrane potential for transport of glutamate into the vesicle, whereas VGLUT2 depends on both the electrical potential and the pH gradient (Bai et al., 2001). VGLUT1 and VGLUT2 are expressed by different types of glutamatergic synapses throughout the brain with little overlap. In the spinal cord VGLUT1 is expressed by synapses of primary sensory mechanoreceptors of either skin or muscle (proprioceptors) and in dorsal spino-cerebellar neurons, while VGLUT2 is mostly expressed by spinal interneurons (Todd et al., 2003; Oliveira et al., 2003; Alvarez et al., 2004; Persson et al. 2006; Llewellyn-Smith et al., 2007). Their expression in motoneurons is highly controversial and will be reviewed in detail in Aim 1. The final known transporter (VGLUT3) was identified from the human genome database by its sequence analogy to VGLUT1 and VGLUT2. VGLUT3 has a more restricted expression pattern and is mainly expressed in cholinergic and serotonergic neurons (Fremeau et al., 2002; Gras et al., 2002; Schäfer et al., 2002).
Interestingly, it was later found that some GABAergic neurons in the brainstem also contain VGLUT3 and co-release glutamate (Stornetta et al., 2005). No neurons in the spinal cord or dorsal root ganglia express transcripts for VGLUT3 (Oliveira et al., 2003). Therefore spinal cord VGLUT3 must originate from descending axons, but their exact distribution has not been thoroughly studied.

Before the cloning of the VGLUTs other methods were employed to identify glutamatergic synapses. For example, the presence of glutamate, glutaminase and excitatory amino acid reuptake transporters have been used to identify glutamatergic primary afferent terminals in the spinal cord (Barbaresi et al., 1985; De Biasi and Rustioni, 1988, 1990; Battaglia and Rustioni, 1988; Broman et al., 1993; Valtschanoff et al., 1994). High levels of glutamate have also been identified in motoneurons and their synapses (Shupliakov et al., 1993; Ottersen & Storm-Mathisen, 1984), as well as glutamate plasma membrane transporters (Meister et al., 1993). However, again due to their widespread distribution and roles in metabolic processes it has been difficult to determine if the presence of glutamate or their transporters in motoneuron cell bodies is indicative of a role in neurotransmission or is evidence for high metabolic activity.

VGLUTs have become broadly accepted as the best available “markers” of glutamatergic synapses. Immunocytochemical studies showed that they are accumulated on synaptic vesicles and different isoforms are distributed to specific glutamatergic pathways (for review, Özkan & Ueda, 1998). VGLUT2 knockouts are fatal at birth because of a lack of excitatory synapses in the brain centers that regulate respiration. VGLUT1 knockouts survive for up to 3 weeks postnatally but neurotransmission from VGLUT1 synapses (for example hippocampus) is abolished following birth (Wojcik et
al., 2004; Moechars et al., 2006; Wallén-Mackenzie et al., 2006). Knockout of VGLUT3 leads to hearing impairments, due to a loss of VGLUT3 in cochlear inner hair cells that release glutamate onto auditory sensory neurons (Ruel et al., 2008). Altogether this indicates that VGLUT expression is sufficient for synaptic vesicular release of glutamate and its loss severely reduces or eliminates glutamatergic neurotransmission. Each VGLUT isoform is expressed by specific subsets of synapses and the loss of one isoform is rarely compensated by others.

**Other possible vesicular excitatory amino acid transporters**

Whereas VGLUTs specifically transport glutamate, the first protein with putative aspartate vesicular transport activity has only recently been described (Miyaji et al., 2008). VGLUTs are part of the SLC/type I phosphate transporter family (Reimer & Edwards, 2004) and many of its members have yet to be defined functionally. Recently, the sialin transporter (SLC17A5) was shown to transport aspartate into vesicles and was localized in synapses (Miyaji et al., 2008). Sialin is better known as a lysosomal transporter that is mutated in sialic acid storage diseases that result in mental retardation and impaired muscle tone and strength (Sagne and Gasnier, 2008). It is yet unknown if sialin also functions as a generalized vesicular aspartate transporter at excitatory synapses in the brain and spinal cord or if other members of the family are able to transport aspartate into synaptic vesicles as well. Interestingly sialin also transports glutamate (Miyaji et al., 2008) and therefore it can potentially have a “VGLUT” role. At present the exact function of sialin at synapses is unknown.
III. Specific aims

To better understand the nature of central motoneuron synapses on Renshaw cells and the possibility of excitatory amino acid release a series of immunocytochemical experiments at the light and electron microscopy levels were performed to test whether motoneuron synapses on Renshaw cells are aspartergic or glutamatergic and to examine possible mechanisms for vesicle neurotransmitter packaging and release.

Aim 1 tested the hypothesis that motoneurons release glutamate through a VGLUT-dependent mechanism. The presence of all three VGLUT isoforms was examined in motoneuron terminals labeled with either VACHT-immunoreactivity (-IR) or using retrograde tracers applied to the ventral roots to label motoneurons and their synapses contacting Renshaw cells. Motoneuron synaptic boutons did not contain significant amounts of any of the VGLUTs, thus falsifying the original hypothesis. We concluded that aspartate, an excitatory amino acid neurotransmitter that is accumulated into synaptic vesicles independent of any of the VGLUTs, might be enriched in the synaptic vesicles of the motoneuron axons. This hypothesis was tested in Aim 3.

Aim 2 tested the hypothesis that the Hb9::EGFP transgenic mouse might be a valuable genetic labeling model to identify motoneuron axons and synapses in the spinal cord. Interpretation of spinal cord synapses labeled with cholinergic markers is complicated by the expression of the same cholinergic markers by some interneurons (which themselves could be glutamatergic) in addition to motoneurons. Immunocytochemical studies in the Hb9::EGFP mouse allowed us to conclude that the large majority of, if not all, VACHT-IR synapses on Renshaw cells originate from motoneurons. In conclusion, VACHT immunolabeling in combination with recognition
of the postsynaptic target as part of a Renshaw cell are adequate criteria for identification of motor axon synapses.

**Aim 3** tested the hypothesis that aspartate was enriched in motor axon terminals compared to glutamate. Because no identified vesicular transporter for aspartate was known at the time, we planned quantitative electron microscopy experiments to directly analyze whether aspartate or glutamate was present at high levels in motor axon synapses. To identify motor axon synapses we used the criteria developed in Aim 2. Preembedded immunolabeling was used to immunolabel VACHT-IR synapses on calbindin-containing dendritic profiles of postsynaptic Renshaw cells. This was combined with postembedding colloidal gold quantitative analyses of aspartate- and glutamate-IR in VACHT-IR motor axon synapses. The results suggest that motor axon synapses on Renshaw cells have a high content of aspartate-IR, whereas glutamate-IR is also enriched but at a lower level and displays a higher variability from synapse to synapse.

**Aim 4** tested the hypothesis that novel members of the solute carrier (SLC) family might be enriched in the synaptic terminals of motoneurons and constitutes a putative novel transport system to accumulate aspartate and/or glutamate inside synaptic vesicles. We tested two different members of this family. SLC10A4 is an orphan transporter highly expressed by cholinergic neurons including spinal motoneurons (Geyer et al., 2008). SLC17A5, or sialin, is the first postulated synaptic vesicle aspartate transporter (Miyaji et al., 2008). Using immunofluorescence methods we demonstrated expression of both proteins in spinal motoneurons, however only SLC10A4 is accumulated in motor axon synapses presynaptic to Renshaw cells.
IV. Aim 1: Characterization of vesicular glutamate transporter (VGLUT) presence at central motoneuron synapses

Rationale

Acetylcholine, the first discovered excitatory neurotransmitter (Dale, 1914), has been thought for many years to be the only neurotransmitter of spinal motoneurons (Dale et al., 1936; Fatt & Katz, 1952). However, there is a growing amount of evidence for a non-cholinergic effect at motoneuron synapses (see background). Further experiments with our collaborators (Mentis et al., 2005), as well as findings by other groups (Nishimaru et al., 2005; LaMotte d’Incamps & Ascher, 2008), demonstrated that NMDA and AMPA receptors are activated postsynaptically on Renshaw cells following motoneuron axon stimulation in the ventral roots.

Therefore in this aim we sought to demonstrate that motor axon synapses on Renshaw cells display a glutamatergic phenotype by analyzing immunocytochemically the presence of the glutamatergic release machinery in the motor axon synaptic boutons. The detection of VGLUT expression at a synaptic terminal indicates that the bouton is glutamatergic (Bellocchio et al., 2000; Herzog et al., 2001; Fremeau et al., 2002, Varoqui et al., 2002). The expression patterns of the three VGLUT isoforms (VGLUT1, VGLUT2, and VGLUT3) have been characterized in the adult spinal cord of rodents and cats, but there are some remaining controversies. There is a general agreement that VGLUT1 is expressed in synapses from cutaneous and muscle mechanoreceptor sensory afferents and by descending corticospinal fibers (Todd et al., 2003; Oliveira et al., 2003; Li et al., 2003; Alvarez et al., 2004; Landry et al., 2004; Wu et al., 2004; Persson et al., 2006), and that boutons with VGLUT2 content arise mostly from glutamatergic
interneurons within the spinal cord (Todd et al., 2003; Oliveira et al., 2003). It has also been suggested that VGLUT2 is present in some sensory afferents, mainly small caliber C-fibers ending in lamina I and II (Li et al., 2003; Landry et al., 2004), however this remains controversial with most studies reporting low or no VGLUT2 in sensory fibers (see Todd et al., 2003; Oliveira et al., 2003; Alvarez et al., 2004; Wu et al., 2004; Morris et al., 2005). One study concluded that significant subpopulations of small nociceptive afferents are not glutamatergic (Morris et al., 2005). New data and a review of previous studies suggested that very low levels of expression (only detected with significant amplification in dorsal root ganglia) and transport might in part explain previous discrepancies in detection (Brumovsky et al., 2007). VGLUT3 fibers in the spinal cord have been little studied but are thought to arise from supraspinal sources since VGLUT3 expressing cells are undetectable in spinal cord and dorsal root ganglia using \textit{in situ} hybridization (Oliveira et al., 2003). We hypothesized that if motoneurons release glutamate, then their synaptic terminals must contain a VGLUT, most likely VGLUT2.

We investigated whether VGLUT-immunoreactivity was present at motor axon synapses identified immunocytochemically by their VACChT content or by retrograde tracing. Because \textit{in situ} hybridization studies were in disagreement about whether motoneurons expressed little or no levels of VGLUTs (see Oliveira et al., 2003; Kullander et al., 2003; Landry et al., 2004; Herzog et al., 2004; Nishimaru et al., 2005; Llewellyn-Smith et al., 2007) we used amplification methods to reveal even the smallest quantities of VGLUT-IR in motor axon terminals. The results falsified our original hypothesis. No evidence for VGLUTs was found in motor axon synapses. The results were published in Mentis et al. (2005).
Materials and methods

Animals, fixation, sectioning

All animals in these studies and in the following aims were used in accordance with Wright State University’s Laboratory Animal Use Committee and NIH guidelines. C57/Black mice and Sprague Dawley rats were used. Mice and rats of different ages postnatal day 5-20 (P5-20) were anesthetized with pentobarbital (Nembutal 70mg/kg i.p.) and transcardially perfused, first with a cold vascular rinse (0.01M phosphate buffer with 137mM NaCl, 3.4mM KCl, and 6mM NaHCO₃, pH 7.3) followed with fixative containing 4% paraformaldehyde (Fisher Scientific, Hanover Park, IL) in 0.1M phosphate buffer (PB). The spinal cords were then removed and post-fixed for four hours. The spinal cord tissue was cryoprotected in a 15% sucrose solution in 0.01M phosphate buffered saline (0.9%) (PBS) at least overnight at 4°C prior to being cut into 20 µm sections using a cryostat or at 50 µm using a sliding freezing microtome. In the case of embryonic mice, the pregnant mouse was transcardially perfused in the same manner as described above. After perfusion, a large incision was made into the abdominal cavity and the embryos were removed, taken from their amniotic sacs and placed in fixative at least overnight, and then moved to a 15% sucrose solution in PBS as described above. Spinal cords were not removed from the embryo prior to being cut using the cryostat. The entire embryo was placed on the cutting stage in the appropriate orientation to obtain transverse spinal cord sections when cutting. Cryostat sections (20 µm) were collected and processed on gelatin coated slides.
Multiple color immunofluorescence for VGLUT 1, 2 or 3 and VACHT on calbindin-IR Renshaw cells

To determine if glutamate is released from motoneuron terminals dual and triple color immunofluorescent experiments were used to test for VGLUTs-immunoreactivity at motoneuron contacts on Renshaw cells. VACHT is a marker of cholinergic synapses in the spinal cord ventral horn and has been used previously to identify motor axon contacts on calbindin-IR Renshaw cells (Alvarez et al., 1999). Colocalization of VGLUT and VACHT in the region containing Renshaw cells and their dendrites could suggest probable co-release of glutamate and acetylcholine from the same synapse.

After rinsing the spinal cord sections in PBS, they were blocked in 10% normal horse or donkey serum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) diluted in 0.01M PBS with 0.1% Triton-X 100 (TX) for 30 minutes. This was followed by an overnight incubation in combinations of the following primary antibodies: VACHT (goat 1:1000, Chemicon, Temecula, CA), VGLUT1 (guinea pig, 1:1000, Chemicon), VGLUT2 (guinea pig 1:2000, Chemicon), VGLUT3 (rabbit, 1:1000, gift from R. H. Edwards, University of California School of Medicine, San Francisco, CA; and guinea pig 1:1000, Chemicon), and calbindin d28k (rabbit, 1:2000; or mouse, 1:500, Swant, Bellinzona, Switzerland), a marker for Renshaw cells throughout development (Geiman et al., 2000). Cryostat sections were processed on slides. Frozen sections cut on the freezing sliding microtome were processed free-floating. In some experiments VACHT antibodies were combined with one of the VGLUT antibodies for dual immunofluorescent experiments. In others, calbindin d28k antibodies were combined with VACHT and VGLUT antibodies for triple immunofluorescent experiments. All
antibodies were diluted in PBS-TX. Following the primary antibody incubation, the sections were rinsed and incubated in appropriate fluorochrome conjugated secondary antibodies (Cy3, Cy5, and FITC) (Jackson ImmunoResearch Laboratories, Inc.) diluted 1:50 in PBS-TX. After a 2-4 hour incubation in secondary antibodies the sections were rinsed in 0.01M PBS and cover slipped with Vectashield (Vector Laboratories, Inc., Burlingame, CA).

Motor axon retrograde labeling and combination with immunohistochemistry

A retrograde tracer was used to label motoneurons and their processes to determine the presence of VGLUT1-3 or VAChT on varicosities of recurrent motor axon collaterals. Briefly, mice (postnatal day 4 (P4) and P10) and rats (P4 and P14) were rapidly decapitated under deep anesthesia (Nembutal 50 mg/kg, i.p.) and the spinal cords quickly dissected in cold artificial cerebral spinal fluid (ACSF) containing: 128.35mM NaCl, 4mM KCl, 0.58mM Na2PO4.H2O, 21mM NaHCO3, 30mM D-glucose, 0.1mM CaCl2.H2O and 2mM MgSO4.7H2O (Sigma-Aldrich, St. Louis, MO). This solution was perfused with a constant flow of 95% O2 / 5% CO2. Spinal cords in P4 animals were used without further dissections, whereas older animals had their spinal cords hemisected for better perfusion with 95% O2 / 5% CO2. Suction electrodes were loaded with Texas red dextran, 10,000MW (1mg in 6µl ACSF) (Molecular Probes/Invitrogen, Grand Island, NY) and applied to the distal cut end of L4 or L5 ventral roots of the spinal cord that was immobilized on a Sylgard base in a chamber superfused with oxygenated ACSF at ~18ºC. The suction electrodes were left attached to the ventral roots for overnight labeling (16-22 hours). Then, the suction electrodes were removed, the tissue was placed
in 4% paraformaldehyde in 0.1M phosphate buffer for a period of four hours to overnight and stored in a 15% sucrose solution in PBS until cryostat-sectioned and processed for standard immunofluorescence. Retrogradely labeled tissue was kindly provided by Dr. George Mentis (NIH, Bethesda, MD).

**Tyramide amplification**

To increase the immunofluorescence signal of proteins possibly present at very low levels (i.e. VGLUT1-3) we used a tyramide amplification kit (Molecular Probes / Invitrogen) (van Gijlswijk et al., 1997). This process increases the number of binding sites available for fluorochrome attachment at antigenic sites and thus amplifies several-fold the immunocytochemical signal. This technique allows detection of proteins present at very low levels or difficult to detect with standard immunofluorescence. Tissue sections were obtained from either immersion-fixed retrogradely labeled spinal cords or transcardially perfused and fixed spinal cords. The sections were blocked in normal serum as before and incubated in guinea-pig antibody solutions against each of the VGLUTs (dilutions: VGLUT1: 1:1,000; VGLUT2: 1:5,000; VGLUT3: 1:2,000). Following overnight incubation in primary antibodies the sections were rinsed in PBS and incubated in anti-guinea pig biotinylated-SP secondary antibodies (Jackson ImmunoResearch Laboratories) diluted 1:250 in PBS with 0.3% TX for 1 hour. The tissue was then rinsed in a solution of 0.1M Tris, 0.15M NaCl, and 0.05% Tween-20 (Fisher Scientific) (TNT) and blocked in a solution of 0.1M Tris, 0.15M NaCl and 0.5% NEN blocking reagent (TNB) for 30 minutes. Following the blocking step, the tissue was incubated for 30 minutes in streptavidin-HRP at 1:2000 diluted in TNB and then rinsed in
TNT. The signal was amplified using a 1:75 dilution of biotinylated tyramide diluted in the provided amplification diluent for approximately 7 minutes. The sections were then rinsed first in TNT then in TNB and incubated in FITC-conjugated streptavidin diluted 1:200 in TNB. After rinsing in PBS a dual immunofluorescence protocol was performed as describe above to reveal calbindin-and VACHT-immunoreactivities.

**Microscopy and image analysis**

Two confocal microscopes were used for capturing images of the different immunofluorescence experiments: 1) A dual channel Olympus FX Fluoview system was used to analyze preparations with two fluorochromes using laser excitation lines of Argon (488 nm) and Krypton (568 nm); 2) A Leica TCS system was used for imaging triple color images using the Argon 488 nm, a HeNe 543 nm and a HeNe 613 nm excitation lines. Immunolabeled sections were imaged at low (10x or 20x) and high magnification (60x, NA 1.4). Image stacks of optical sections were collected throughout the section thickness with z-steps of 1 or 2 µm (low magnification images) or 0.5 µm (high magnification images). In all cases the preparations were imaged using simultaneous excitation and photomultiplier (PMT) detector settings adjusted to minimize channel cross-talk. Confocal images were analyzed using Image ProPlus 5.1 and Fluoview software. Digital manipulations of images were done only for figures preparation and were kept to a minimum not altering the information content of the images.

**Analysis of VGLUTs and VACHT colocalization**
Analyses were performed using high magnification confocal images of the Renshaw cell region dual immunolabeled for VACHT and each VGLUT. This region is well defined in segments L4 and L5 as a ventral LVII region just medial to LIX and containing the exiting axons of motoneurons (Fig. 1). In dual immunolabeled sections, VGLUT-IR (1, 2 or 3) and VACHT-IR punctae were thresholded, segmented from the image and counted (Fig. 3) to obtain a density estimate of labeled punctae per 100 µm² in single optical sections. Then the image was simultaneously thresholded in both channels and the number or punctae with both immunoreactivities counted. In all thresholds random noise was eliminated by filtering punctae smaller than 0.25 µm in average diameter. Then the percentage of VACHT punctae containing VGLUT immunoreactivity was calculated. At least 3 different ventral horn images were examined in each experiment and at each different age examined (see Table 2).

To determine the amount of random colocalization the VACHT-IR image was shifted in the X-Y plane 5 µm to the left and then re-merged with the original image of the corresponding VGLUT-IR. Following re-merging, segmentation and thresholding were performed as above and the amount of overlap estimated. These experiments were done in P20 spinal cords and compared to colocalization values calculated in properly aligned VACHT-IR and VGLUT-IR images.

**Analysis of retrogradely labeled synaptic varicosities**

Retrograde tracing techniques label motoneuron cell bodies, their axons and dendrites. Threshold segmentation of the images would have given false-positive values by selecting in addition to synaptic varicosities the whole dendritic tree and motoneuron
somata. VACHT-IR punctae in synaptic apposition to motoneuron dendrites and cell bodies are close enough to result in positive colocalization. Therefore, for determining colocalization between retrogradely labeled motor axon varicosities and each of the VGLUTs in the Renshaw cell area we used a line analysis provided in Fluoview software. In this analysis, a line was drawn through each varicosity and the intensity detected by each channel was measured along the drawn line (see Fig. 6). Peak intensity values along the line were then compared to a random sample of background intensities for correction. Background values were estimated by placing the drawn line adjacent to the puncta being measured. We estimated the percentage of retrogradely labeled varicosities containing co-registered peak intensities for VACHT, VGLUT1, VGLUT2, and VGLUT3 at 2 and 4 standard deviations above the average background. Background intensities were normally distributed, therefore 2 and 4 standard deviations represent respectively values with less than a 5% and 0.01% chance of being due to background. We analyzed approximately 50 retrogradely labeled varicosities for each marker (VACHT, VGLUT1, VGLUT2, and VGLUT3) and animal age (see Table 3).
Results

Distribution of different VGLUT isoforms in the Renshaw cell region

Immunofluorescence was used to determine the presence of each of the VGLUTs in the motoneuron and Renshaw cell regions of the spinal cord. The motoneuron pool is located in lamina IX (LIX) and medial to this is the ventral portion of LVII containing Renshaw cells (Fig. 1A). The Renshaw cells are identifiable by their calbindin-IR content and clustering in ventral LVII (see Alvarez & Fyffe, 2007). Motoneurons are identified by their large size and cholinergic content, being immunoreactive for choline acetyltransferase (ChAT) and vesicular acetylcholine transporter (VAChT) (Fig. 1B, C). ChAT-IR densely labels motoneuron somata and can also be seen in a few interneurons outside LIX, as will be described in Aim 2 (Fig. 1B). VAChT-IR labels the somata of motoneurons and cholinergic interneurons less intensely than ChAT-IR. Most VAChT immunoreactivity is associated with punctae representing cholinergic synapses in the axon terminals. VAChT-IR punctae occur at high density in the Renshaw cell area (Fig. 1C) and make contacts with Renshaw cells (Fig. 1D); most varicosities target Renshaw cell dendrites (Fig 1D). Motoneuron somata also receive input from VAChT-IR cholinergic synapses. They represent the so-called C-terminals known to originate from cholinergic interneurons (Miles et al., 2007).

In the postnatal spinal cord VGLUT1-IR and VGLUT2-IR punctae are present throughout the neuropil. In the ventral horn they are dense in both LIX and the LVII Renshaw cell region (Fig. 1E, F). VGLUT1-IR is strongly expressed in the central projections of muscle afferents (see also Todd et al., 2003 and Alvarez et al., 2004). Thus there are numerous large VGLUT1-IR punctae in LIX where these afferents are
contacting motoneurons. Outside LIX the density of VGLUT1-IR gradually decreases toward the more medial LVII and LVIII. Notably, there are fewer VGLUT1-IR punctae in the Renshaw cell area in LVII. The individual VGLUT2-IR punctae are smaller in appearance than VGLUT1-IR punctae, but VGLUT2-IR is denser than VGLUT1-IR throughout the ventral horn including LIX and the Renshaw cell area (Fig. 1F, Table 1).

VGLUT3-IR fibers and varicosities occur at very low density in the mouse and rat spinal cord. Moreover, VGLUT3-IR showed species differences between mouse and rat tissue in their distribution. In both mouse and rat spinal cord, VGLUT3-IR was observed in the superficial lamina of the dorsal horn (Fig. 2A, C), although staining in the mouse was more intense than in rat. In the ventral horn VGLUT3-IR axons were only detected in rat spinal cord tissue sections (Fig. 2B, D). These axons were sparsely distributed (Fig. 2D), but a portion crossed through the Renshaw cell area in LVII (Fig. 2D, inset).

**VACHT-IR boutons in the Renshaw cell region lack VGLUT immunoreactivities**

Spinal cord sections were dual immunolabeled with antibodies against VACHT and each one of the VGLUT isoforms to test for dual colocalization as explained in the methods and in figure 3. Tyramide amplification was used to increase detection of VGLUTs even if they were present at very low levels. Image thresholding of VGLUT1 & 3-IR and VACHT-IR punctae in the same Renshaw cell regions of P20 spinal cords (VGLUT1, mouse and VGLUT3, rat) showed that these VGLUT isoforms were less dense than VACHT-IR boutons (Fig. 3 and Table 1). VGLUT2-IR punctae were however denser than VACHT-IR boutons in the Renshaw cell area (Fig. 3, Table 1). Using the dual thresholding / segmentation method (Fig. 3) we found at age P20 four points of
colocalization between VGLUT1 and VACHT in a total of 899 VACHT-IR varicosities in the Renshaw cell area (0.35%). No VGLUT2-IR punctae were colocalized with 1,356 VACHT-IR varicosities collected in the Renshaw cell area. VGLUT3 had one IR puncta that colocalized with the 1,097 VACHT-IR punctae sampled in the Renshaw cell area (0.06%) (Tables 1 & 2). In the few instances in which colocalizations were detected, frequently we could demonstrate that this was due to superimposition in the z-axis of independent punctae (Fig. 4). We concluded that VACHT-IR and VGLUT-IR do not colocalize in the Renshaw cell region of P20 rodent spinal cords.

Because most analyses of motor axon non-cholinergic actions on Renshaw cells were performed in neonatal preparations, we then questioned if it was possible that there was a developmental loss of VGLUT expression in VACHT-IR synapses over postnatal developmental time. Therefore we examined VGLUT1 & VGLUT2 at earlier ages. At embryonic day 15 (E15) only VGLUT2 was examined, as VGLUT1 and VGLUT3 are not found in the spinal cord at this age. VGLUT3 is not present in these younger ages most likely because it is contained in late arriving descending inputs (Oliveira et al., 2003). Primary sensory afferents are invading the spinal cord at this time and VGLUT1 has not yet up regulated in these relatively immature axons (Mentis et al., 2006). Using the same methods to determine colocalization, three points of colocalization between VACHT and VGLUT2 were found in 295 VACHT-IR varicosities in the embryonic Renshaw cell area (1.02%). At P5, five points of colocalization between VGLUT2 and VACHT in 323 VACHT-IR varicosities in the Renshaw cell area (1.55%) were observed. While VGLUT1 and VACHT had five points of colocalization in 330 VACHT-IR varicosities in the P5 Renshaw cell area (1.52%). In conclusion, as observed in P20
animals, the E15 and P5 spinal cords also lacked significant amounts of colocalization between VGLUT-IR and VACHT-IR punctae in the Renshaw cell area (Table 2).

It is possible that this small level of colocalization occurs because of random superimpositions. To test the amount of random overlap that could be expected at these densities of immunolabeling we shifted the VACHT-IR image by 5 µm over the VGLUT-IR image. The amount of colocalization observed following this shift was greater than the colocalization observed in the matched images. VGLUT1-IR exhibited 8 points of colocalization in 664 VACHT-IR varicosities in the shifted image (1.2%). In shifted images of VGLUT2-IR and VACHT-IR there were 236 points of colocalization for 1,101 VACHT-IR varicosities (21.4%). In the shifted images of VGLUT3-IR and VACHT-IR samples there were 21 points of colocalization observed in 844 VACHT-IR varicosities (2.4%). The percentages of random overlap in shifted images parallel the different densities of VGLUT1, VGLUT2 and VGLUT3 and were always larger than the actual colocalization amounts estimated in the appropriately aligned images. This suggests that VGLUTs are actually excluded from VACHT punctae and that the small percentages of colocalization are within the amount that could be expected by random overlap. This could either be due to superimposition of two labeled punctae at different levels in the z-axis within the focal depth of the X60 objective optical section (N.A. 1.35: approximately 0.85-0.95 µm) or because partial superimposition of immunofluorescence signals in the x/y plane when two boutons are adjacent to each other.
Retrogradely labeled synaptic varicosities contain VACHT but not VGLUTs

After determining that there was a lack of colocalization in the Renshaw cell area between VACHT-IR punctae and each of the VGLUTs we used a retrograde tracer to label motoneurons and their processes (Fig. 5). This was done to rule out the possibility that VACHT and VGLUTs are localized in different motor axons terminals from the same or different motoneurons, as proposed by Herzog et al. (2004). The analyses also confirmed that VACHT-IR contacts on Renshaw cells originate from motor axon synapses. VACHT-IR boutons were bright and dense in retrogradely labeled varicosities in the Renshaw cell area (Fig. 5A-E). Using the line analysis described in the methods section and in figure 5 in two mice of different ages (one P4, one P10) we estimated that 87% (at P4) and 79% (at P10) of retrogradely labeled motoneuron varicosities contained respectively VACHT-IR at 2 or 4 standard deviations above the measured average background immunoreactivity estimated. In contrast, VGLUT2-IR was detected 2 standard deviations above background in only 2% of varicosities at P10 and in no varicosities above 4 standard deviations or in P4 spinal cords. Similarly, no varicosities exhibited VGLUT3 immunoreactivity with intensity above 2 standard deviation of background at any age. VGLUT1 was detected in 2-4% of varicosities at different ages or threshold levels above background (Table 3). These small amounts of colocalization are similar to those found in the VGLUT / VACHT colocalization study and might also be due to random appositions and superimpositions in respectively the x-y and z axes.
In conclusion, we were unable to demonstrate VGLUT-immunoreactivity inside motor axon synapses using different labeling methods and amplification techniques in developing or mature spinal cords.
Figure 1: Location of cellular and synaptic elements of the recurrent inhibitory circuit and distribution of VGLUT1 and VGLUT2 immunoreactivities in the ventral horn. A) Schematic of the spinal cord indicating the location of motoneurons (α-MNs) and Renshaw cells (RC) and their reciprocal connections. B) Single optical plane confocal microscope image showing ChAT-IR motoneurons (Cy3, red) and calbindin-IR Renshaw cells (Cy5, blue) in a region of the ventral horn that approximately corresponds to that drawn in A. C) Two-dimensional projection of a stack of confocal images throughout the 50 µm-thick section immunolabeled for VAChT (Cy3, red) and calbindin (Cy5, blue). VAChT-IR terminals are concentrated around motoneuron (MN) somata and in relation with calbindin-IR Renshaw cells (outlined by a dashed line). VAChT-IR terminals around motoneurons are of interneuronal origin and are denominated C-terminals. VAChT-IR boutons associated with Renshaw cells are believe to originate from motor axons (Alvarez et al., 1999). D) High magnification confocal image of a calbindin-IR Renshaw cell (blue) receiving contacts from VAChT-IR boutons (red) along its dendrite and on the cell soma. E) VGLUT1 immunoreactivity in the ventral horn is very dense around motoneurons in LIX and its density somewhat diminishes in the Renshaw cell area (boxed region). F) VGLUT2 immunoreactivity in the ventral horn is denser than VGLUT1 and its density does not diminish in the Renshaw cell area (boxed region) as much as VGLUT1. Scale bars in B, C, and F are 100 µm, D, 10 µm. E is at the same magnification than F.
**Figure 2: VGLUT3-immunoreactivity in the spinal cord of the mouse and rat.**  

A) Dorsal horn of a P5 mouse spinal cord exhibits intense VGLUT3-IR in lamina II.  
B) The ventral horn of P5 mouse spinal cord lacks VGLUT3-IR (similar in older ages, not shown).  
C) Lamina II in the adult rat spinal cord contained weak VGLUT3 immunoreactivity.  
D) In contrast to the mouse there are sparsely distributed VGLUT3-IR fibers in the ventral horn of the rat spinal cord and some crossed the Renshaw cell region. The inset shows at higher magnification the region boxed in D. Scale bar in D is 200 µm. All panels are at the same magnification.
Figure 3: Lack of VGLUT-IR colocalization with VAChT-IR in the Renshaw cell area. A-C) High magnification image of a single confocal optical plane in the Renshaw cell region showing VGLUT1 (A, Cy3, red), VAChT (B, FITC, green) and both immunoreactivities superimposed (C). There was barely any colocalization. D-F) Binary images obtained after thresholding and segmenting the VGLUT1 (D) or VAChT (E) signals and VGLUT1/VAChT colocalization (F). The number of punctae from these binary images was counted by the software to obtain the data shown in Tables 1 and 2. G-I) High magnification images as described before, but for a field immunostained for VGLUT2 (G) and VAChT (H). Both signals superimposed are shown in I. J-K) Thresholding and segmentation of the images also show little colocalization between VGLUT2 and VAChT (I). Scale bar in L is 100 µm. All panels at the same magnification.
Figure 4: Superimposition in the z-axis can result in false colocalization values. A) VGLUT (FITC, green) and VAChT-IR (Cy3, red) punctae in the Renshaw cell area of the ventral horn occasionally appears to co-localize (arrow). This appearance of colocalization is confirmed in the binary image after thresholding and segmentation for both immunoreactivities (E, arrow). B-D) Fluoview software analysis of this colocalization through two perpendicular planes in the z-axis (C, D, are orthogonal views of the crossing lines in B) indicate that the red (VAChT) and green (VGLUT1) clusters are in fact superimposed in the z-axis and not colocalized. Thus, there is not true colocalization, but rather two separate VGLUT and VAChT-IR punctae in close proximity and superimposed with each other within the tissue section thickness (50 µm).
Figure 5: VACHT, VGLUT1, VGLUT2, and VGLUT3 immunoreactivity in retrogradely labeled motor axons and contacting Renshaw cells. A) Motoneurons in a P10 mouse spinal cord are retrogradely labeled with Texas Red dextran (TX Red Dxt, red) and appear intensely labeled in their somata, dendrites and axons exiting the ventral roots. B, C) Retrogradely labeled motor axon collaterals displayed many varicosities (B) that contained VACHT-IR (FITC, green; colocalization, yellow (arrowheads)). D, E) In a triple immunolabeled section, calbindin-IR Renshaw cells (Cy5, blue, arrowheads in D) in ventral LVII were contacted by retrogradely labeled motoneuron varicosities (Cy3, red, arrows in D indicate motoneuron somata) that contained VACHT-IR (FITC, green). F, G, H) In P10 mice, there was no significant colocalization (arrow in H) between retrogradely labeled motoneuron varicosities (Texas red) and any of the VGLUTs (FITC, green). I, J1, J2) At high magnification, VGLUT1-IR (I) and VGLUT 2-IR (J) were observed contacting Renshaw cells (Cy5, blue), but never colocalized with VACHT-IR contacts (Cy3, red) on these same neurons. K) Renshaw cells (Cy3, red) received very few contacts from VGLUT3-IR (FITC, green, arrows) boutons and these terminals did not colocalize with VACHT. Scale bars: A, 200 µm; B, C, 10 µm; D, 50 µm; E, 10 µm; F-H, 10 µm; I, 20 µm; J, 10 µm; K, 20 µm.
Figure 6: Line analysis for detection of VGLUT-IR and VACht-IR in retrogradely labeled motor axons. A,B,E,F) Motor axons and varicosities retrogradely labeled with Texas Red dextran (red, A,E) and respectively immunolabeled for VACht (B) or VGLUT2 (F). C, G) Superimposition of retrograde labeling and VACht (C) or VGLUT2 (G). A line is traced through the center of the varicosities to measure fluorescence intensity in both channels. D, H) Fluorescence intensity plots along the lines. In both cases the red channel fluorescence (retrograde labeling) saturated at 4095 (12-bit images). While VACht-immunolabeling intensity (green channel, D) also peaks and co-distributes within the fluorescence that defines the varicosity (red, D), VGLUT2 fluorescence intensity (green, H) was at background levels, i.e., no different inside or outside the varicosity (red, H). Scale bar in G is 5 µm. Panels A, B, C, E and F are at the same magnification.
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<th>VGLUT</th>
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<td>Mean ± S.D.</td>
<td>Mean ± S.D.</td>
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<td>VGLUT1, P20 mouse</td>
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<td>0.20 ± 0.06</td>
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<tr>
<td>VGLUT2, P20 mouse</td>
<td>0.47 ± 0.20</td>
<td>1.18 ± 3.39</td>
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<td>VGLUT3, P20 rat</td>
<td>0.38 ± 0.15</td>
<td>0.17 ± 0.02</td>
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Sample region: 23,900 µm² in each of 12 optical sections
Total area: 12 x 23,900 µm² = 286,800 µm² of Renshaw cell area
3 optical sections sampled in 4 ventral horns for each transporter
Table 2: VGLUT colocalization with VACHT in the Renshaw cell area.

<table>
<thead>
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<th></th>
<th>E15</th>
<th>P5</th>
<th>P20</th>
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<tbody>
<tr>
<td>VGLUT1 (mouse)</td>
<td>NA</td>
<td>1.55% (n=330)</td>
<td>0.35% (n=899)</td>
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<tr>
<td>VGLUT2 (mouse)</td>
<td>1.02% (n=295)</td>
<td>1.52% (n=323)</td>
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<td>VGLUT3 (rat)</td>
<td>NA</td>
<td>NA</td>
<td>0.06% (n=1097)</td>
</tr>
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NA (not applicable), because the specific VGLUT isoform is not present at this age
Table 3: VAChT and VGLUT inside retrogradely labeled motor axon varicosities.

<table>
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<tr>
<th>Animal &amp; age</th>
<th>N (number of varicosities)</th>
<th>Labeling 2 SD above background</th>
<th>Labeling 4 SD above background</th>
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<tbody>
<tr>
<td>VAChT P4 mouse</td>
<td>50</td>
<td>82%</td>
<td>80%</td>
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<tr>
<td>VAChT P10 mouse</td>
<td>51</td>
<td>90%</td>
<td>78%</td>
</tr>
<tr>
<td>VGLUT1 P4 mouse</td>
<td>50</td>
<td>2%</td>
<td>0%</td>
</tr>
<tr>
<td>VGLUT1 P10 mouse</td>
<td>49</td>
<td>4%</td>
<td>4%</td>
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<td>50</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>VGLUT2 P10 mouse</td>
<td>50</td>
<td>2%</td>
<td>0%</td>
</tr>
<tr>
<td>VGLUT3 P4 rat</td>
<td>50</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>VGLUT3 P14 rat</td>
<td>50</td>
<td>0%</td>
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Discussion

VGLUT1 and VGLUT2 are expressed in the spinal cord ventral horn of young mice with specific patterns similar to those demonstrated in the adult rat (Todd et al., 2003; Oliveira et al., 2003; Li et al., 2003; Alvarez et al., 2004; Landry et al., 2004; Wu et al., 2004; Persson et al., 2006; Brumovsky et al., 2007; Llewellyn-Smith et al., 2007). VGLUT3 expression in the spinal cord is much weaker than VGLUT1 and VGLUT2 and differs between mouse and rat spinal cord. Only in the rat were significant amounts of VGLUT3-IR axons found in the ventral horn. Although labeling of VGLUT1 and VGLUT2 were dense throughout the ventral horn, including the Renshaw cell area, the immunocytochemical experiments failed to show significant amounts of VGLUT-IR in either VACHT-IR or retrogradely labeled motoneuron synaptic varicosities. The small percentages of colocalization between VACHT-IR and VGLUT-IR were likely not true colocalization but due to immunoreactive punctae in close proximity to each other or random superimposition in the z-axis. Furthermore, small amounts of VGLUT-IR colocalized with retrogradely labeled motor axons may not have been at synapses but at labeled motoneuron processes located in the Renshaw cell area or running perpendicularly to the calbindin-IR process, but not in direct contact with the Renshaw cell itself. It is doubtful that the small percentages of colocalization found, even if a few represent true colocalization at motoneuron axon terminals, could support the significant functional co-release of acetylcholine and excitatory amino acids described by Mentis et al. (2005) and Nishimaru et al. (2005) and later confirmed by Lamotte D’Incamps and Ascher (2008).
Whereas we were unable to detect VGLUT-IR in motoneurons, others have used *in situ* hybridization techniques to suggest VGLUT1 and VGLUT2 expression in motoneurons (Landry et al., 2004; Herzog et al., 2004). However, close examination of the *in situ* hybridization published data shows a lack of contrast between motoneurons putatively expressing VGLUT mRNA signals and background VGLUT mRNA labeling. Furthermore, the immunohistochemistry evidence in Herzog et al. (2004) showed extensive presence of VGLUTs in the soma and dendrites of motoneurons (data not confirmed by other authors: Todd et al., 2003; Oliveira et al., 2003; Alvarez et al., 2004), but surprisingly they were unable to detect VGLUT-IR in axon terminals containing VAChT-IR in the Renshaw cell area or at the neuromuscular junction. Nonetheless they show evidence of VGLUT content in a single example of an intracellularly filled motoneuron process that was interpreted as an axon without further corroborating information. These data led Herzog and colleagues to speculate about the possibility that different collaterals of the same axon contained either VGLUT2 or VAChT. To our knowledge there is no molecular basis or mechanism for such selective distribution of synaptic molecules in axons. The studies suggesting the presence of VGLUTs in motoneurons are therefore very controversial. Most other reports failed to localize VGLUT1 and VGLUT2 mRNA to the motoneurons (Oliveira et al., 2003; Kullander et al., 2003; Llewellyn-Smith et al., 2007).

One group first reported that VGLUT2 mRNA was undetectable in motoneurons (Kullander et al., 2003), but later suggested its presence claiming enhanced sensitivity (Nishimaru et al., 2005). Nevertheless, in agreement with our data, in more than a thousand samples of VAChT-IR punctae in the Renshaw cell area; this group was only
able to colocalize three VACHT-IR punctae with VGLUT2-IR (Nishimaru et al., 2005). Moreover, in an image depicting their in situ hybridization data the mRNA labeling of motoneurons is extremely weak as compared to the strongly labeled interneuron somata throughout the ventral horn (Nishimaru et al., 2005). It was argued that the lack of evidence for VGLUT-IR at central motoneuron synapses might be due to low levels and a failure of detection. It is possible that the sensitivity of our immunostaining was less than that of other groups. However, this seems unlikely as tyramide amplification was used to amplify even the weakest signal and all groups agree on the lack of colocalization between VGLUTs and VACHT. Furthermore, a recent study performed in both the cat and rat shows that VGLUT1-IR and VGLUT2-IR are not detectable in VACHT-IR boutons contacting rat Renshaw cells or inside single intracellularly labeled cat intraspinal motor axons (Liu et al., 2009). However this group did report VGLUT2-IR in a population of ventral horn cholinergic VACHT-IR terminals but outside the area containing Renshaw cells. Most of our data indicating a lack of VGLUT-IR in motoneuron terminals contacting Renshaw cells was published in Mentis et al., (2005) and these recent results by an independent group seem to confirm our conclusions in this paper and in this aim.

The lack of strong VGLUT-IR at motoneuron synapses suggests alternative hypotheses for excitatory amino acid release. One possibility is that excitatory amino acids other than glutamate, for example, aspartate, are being released from motoneuron terminals. Aspartate does not require any of the known VGLUTs for transport into vesicles for synaptic release (Bellocchio et al., 2000; Herzog et al., 2001; Fremeau et al., 2002, Varoqui et al., 2002). Recently, an independent vesicular transporter for aspartate
has been proposed (Miyaji et al., 2008). The electrophysiological data indicating the activation of NMDA and AMPA receptors (Mentis et al., 2005 and Nishimaru et al., 2005; LaMotte d’Incamps & Ascher, 2008) in Renshaw cells in response to motor axon stimulation allow for the possibility for aspartate being released from the motoneuron terminals. Aspartate, similar to glutamate, binds and opens NMDA receptors (Patneau & Mayer, 1990; Curras & Dingledine, 1992). Interestingly, aspartate is less effective on AMPA receptors and this might explain the relatively small peak amplitudes of AMPA synaptic currents compared to NMDA (LaMotte d’Incamps & Ascher, 2008).

Another possibility for a lack of detectable VGLUTs at motoneuron synapses is that there is another yet unknown transporter that could be moving glutamate and aspartate into vesicles for release at the synapse. In other areas of the spinal cord where glutamate is suggested to be released, for example at the central terminals of nociceptors, only weak VGLUT expression is observed (Todd, et al., 2003; Alvarez et al, 2004). Similar findings led one group to claim that glutamate is not released at nociceptive terminals (Morris et al., 2005), but this possibility has been recently challenged based on new data and careful review of discrepancies in the literature (Brumovsky et al., 2007).

The lack of correlation between VGLUT content and the physiologically demonstrable excitatory amino acid releases from nociceptive and motor axon synapses in the spinal cord tests the generalized view that VGLUTs are the only mechanism of excitatory amino acid (glutamate or aspartate) accumulation in synaptic vesicles. Recently a putative aspartate transporter with glutamate transport capability has been detected in selected pools of synaptic vesicles (Miyaji et al., 2008). This transporter will be reviewed more thoroughly in following aims.
It is unlikely that a closely related VGLUT remains to be identified because the discovery and description of VGLUT3 by three independent groups included the thorough analysis of genomic sequences in several different species (Gras et al., 2002; Schaffer et al., 2002; Fremeau et al., 2002). However, VGLUTs are part of a larger more extensive family of transporters with many members that have not yet been functionally identified. One of these functionally unidentified transporters may be capable of transporting both excitatory amino acids into vesicles. The recently reported aspartate transporter is such a candidate and a distant relative of VGLUTs within the superfamily of solute ligand carriers (SLCs).

Alternative packaging mechanisms for transporting excitatory amino acids into vesicles makes it possible that both excitatory amino acids are co-stored and co-released in synaptic vesicles of motoneuron terminals in a VGLUT-independent manner. There is good evidence in the CNS for the co-release of aspartate and glutamate from the same synapse (Gunderson et al., 1998; Gunderson et al., 2004). The lack of detectable VGLUTs at motoneuron synapses therefore does not exclude the possibility of excitatory amino acid release from these synapses. However it is necessary that motor axon synaptic bouton have high levels of glutamate and or aspartate to be substrates for a novel putative transport mechanism for excitatory amino acids. Further experiments are therefore needed to test whether glutamate and/or aspartate are accumulated in intraspinal motoneuron synapses on Renshaw cells. These experiments are explained in Aim 3.
V. Aim 2: Characterization of the Hb9::EGFP mouse model for labeling motoneuron synapses contacting Renshaw cells

Rationale

Lack of VGLUT labeling of motoneuron terminals makes it necessary to examine directly the excitatory amino acid content of motoneuron synapses contacting Renshaw cells. If glutamate and/or aspartate are indeed accumulated in synaptic terminals, even through a VGLUT-independent process, then we should be able to demonstrate enrichment using immunohistochemical techniques. Preliminary immunofluorescence experiments (not shown) tested excitatory amino acid immunolabeling and showed that the quality of glutamate- and aspartate-IR was fixation sensitive, similar to previous reports (Waerhaug & Ottersen, 1993; Larsson et al., 2001). To optimally reveal glutamate and aspartate in tissue sections it is necessary to fix the tissue rapidly through perfusion and with strong fixatives that include high concentrations of glutaraldehyde. Unfortunately the in vitro retrograde tracing techniques used in Aim 1 to identify motor axons are not compatible with this fixation regimen. This is because fixation is performed by immersion of the isolated spinal cord after long in vitro incubations (usually overnight) that are necessary for retrograde transport, but that likely wash away much of the glutamate and aspartate in the tissue. In one experiment in which motor axons were retrogradely labeled with biocytin applied to a ventral root using the method described in Aim 1 and then immersion fixed and processed for electron microscopy we obtained good retrograde labeling of motoneurons but poor ultrastructure preservation and barely any glutamate immunoreactivity. We believe this was the result of poor fixation. Further attempts to improve fixation speed and penetration with microwave
irradiation did not result in significant improvements. We therefore concluded that this method of motor axon labeling cannot be combined with optimal glutamate and aspartate immunolabeling.

Alternatively, VAChT-IR can be used to label motor axon synapses on Renshaw cells (Alvarez et al., 1999). Because of their relatively large size, on average, the majority of these contacts were considered to originate from motor axons; however the presence of some cholinergic interneurons in the spinal cord (Barber et al., 1984; Phelps et al., 1984; Miles et al., 2007) could confound interpretation of specific individual VAChT-IR contacts if these interneurons were also presynaptic to Renshaw cells. Therefore we sought a different labeling method for identification of intraspinal motor axons that could be used in combination with transcardial perfusion fixation and allow optimal immunolabeling conditions for glutamate and aspartate antibodies. In doing the analysis we also tried to confirm whether cholinergic interneurons are an additional significant source of cholinergic inputs on Renshaw cells.

In the recently developed Hb9::EGFP transgenic mouse line motoneurons and their processes express enhanced green fluorescent protein (Wichterle et al., 2002). We predicted that EGFP labeling likely extends into motor axons and their synapses providing a good marker model that could also be perfusion fixed and combined with excitatory amino acid immunocytochemistry. In Hb9::EGFP animals EGFP expression is dependent on regulatory elements of the Hb9 gene, which encodes a transcription factor that suppresses interneuronal phenotypic differentiation and is essential for motoneuron development (Arber et al., 1999; Wichterle et al., 2002). Thus the Hb9 transcription factor is highly expressed in motoneurons; however it is also expressed in a population of
interneurons (Hinckley et al., 2005; Wilson et al., 2005). Moreover EGFP expression from this transgene appears to be somewhat “leaky” resulting in its ectopic expression in other interneurons that do not express the Hb9 transcription factor. Since the development of these mice is relatively new, the expression of EGFP in this model has yet to be fully characterized. Specifically, the developmental regulation of expression of the transgene and the trafficking of EGFP into the motor axons is unknown. In addition whether all EGFP axons contacting Renshaw cells are originating from motor axons or some could belong to interneurons was unknown. A thorough examination of EGFP expression in the spinal cord of Hb9::EGFP mice is therefore required to determine the suitability of this model for examining motoneuron contacts on Renshaw cells.

The analyses performed in this aim suggested that VAChT and EGFP contacts on Renshaw cells are both the same population of synaptic boutons and originate from motoneurons. In other words, motoneurons are the sole significant source of cholinergic inputs onto Renshaw cells and in Hb9::EGFP mice EGFP synaptic varicosities targeting Renshaw cells originate from motor axons. However, EGFP labeling decreases with development while VAChT labeling is always intense in motor axon synapses on Renshaw cells of any age.
Materials and methods

Animals

To obtain the required genotypes Hb9::EGFP male mice were crossed with C57/Black female mice to generate Hb9::EGFP heterozygotes. These animals were bred in the LAR facilities at Wright State University. The pups were tattooed in their paws for identification purposes. Following tattooing tail clips were made prior to age P5 for genotyping using standard PCR. DNA was extracted from the tail clips using Qiagen’s DNeasy kit (Qiagen, Germantown, MD). Genotypes were determined using the following primers for EGFP: (5’ to 3’); GAC CTA AAC GGC CAC AAG TT, and (3’ to 5’); GAA CTC CAG CAG GAC CAT GT. PCR results were obtained using a MyCycler Bio Rad thermocycler with HotMaster Taq DNA Polymerase (Eppendorf Brinkmann Instruments, Inc.) and the following protocol: 5 minutes at 95°C pre-melt step, followed by 35 cycles of 30 seconds 95°C melt, 30 seconds 60°C anneal, and 7 minutes of 72°C extension. PCR products were then visualized using a 2% agarose gel electrophoresis in 1x TBE buffer with ethidium bromide staining. A 500bp PCR product indicates the presence of the Hb9::EGFP transgene. Those animals expressing the appropriate PCR product were fixed via transcardial perfusion and processed for immunofluorescent experiments.

Fixation and sectioning

Hb9::EGFP mice of 5-20 days postnatal age (P5-20) were anesthetized with pentobarbital (Nembutal or Euthasol >70mg/kg i.p.) and transcardially perfused, first with a cold vascular rinse (0.01M phosphate buffer with 137mM NaCl, 3.4mM KCl, and...
6 mM NaHCO3, pH 7.3) followed by fixative containing 4% paraformaldehyde (Fisher Scientific) in 0.1 M phosphate buffer (PB). The spinal cords were then removed and post-fixed for four hours. The spinal cord tissue was cryoprotected in a 15% sucrose solution in 0.01 M PBS at least overnight at 4°C prior to being cut into 20 µm sections using a cryostat.

Neurolucida analyses of EGFP expressing neurons in Hb9::EGFP animals

Immunofluorescent preparations of Hb9::EGFP mice spinal cords were analyzed using a standard epifluorescence Olympus BX51 microscope coupled to the Neurolucida tracing/plotting system (MicroBrightField, Colchester, VT). This system was used to identify the locations of neurons in the ventral horn that expressed EGFP during postnatal development. To enhance EGFP-labeling of neuronal processes, spinal cord sections from Hb9::EGFP mice were immunolabeled using primary antibodies for sheep or rabbit anti-EGFP (1:800, Biogenesis) and revealed with FITC-conjugated secondary antibodies (1:50, Jackson ImmunoResearch). EGFP-labeling was then combined with either guinea pig anti-VAChT (1:1000, Chemicon) to label terminals releasing acetylcholine, goat anti-choline acetyl transferase (ChAT) (1:1000, Chemicon) to define cholinergic neurons and mouse anti-NeuN (1:1000, Chemicon) a generic marker of neurons. Cy3 or Cy5 conjugated secondary antibodies were used to reveal ChAT, VACChT, or NeuN in dual and triple immunofluorescent preparations, similar as in the previous aim. Using the Neurolucida system, the total number of NeuN-IR neurons in the ventral horn was counted and their locations plotted within drawn contours of the spinal cord section and its laminae. This was followed by counting and plotting the total number of EGFP
labeled neurons (categorized into large neurons within lamina IX and smaller interneurons outside of lamina IX). Then, neurons labeled with either VACHT or ChAT were plotted and counted and their colocalization with EGFP determined. In this manner we sought to confirm that the only ChAT or VACHT positive neurons expressing EGFP are motoneurons in lamina IX. Similar analyses were performed in P0, P5, P10, and P20 animals to also examine the developmental regulation of EGFP expression.

**Confocal analysis of Hb9::EGFP axons in the Renshaw cell region**

The purpose of this analysis was to confirm that Hb9::EGFP axons contacting Renshaw cells are cholinergic and therefore originating from motoneurons. In addition, we also analyzed whether all VACHT-IR contacts on Renshaw cells were labeled with EGFP. For this purpose we performed a quadruple immunofluorescent experiment with the following antibodies: sheep anti-EGFP (1:800), guinea pig anti-VACHT (1:1000), mouse anti-calbindin d28k (1:1000), and rabbit anti-SV2B (1:2000, Chemicon). SV2B is a synaptic vesicle glycoprotein, and the antibody was used to identify EGFP synapses in apposition to calbindin-IR Renshaw cells. To avoid cross-reaction between secondary antibodies in quadruple immunofluorescence experiments requiring goat raised anti-rabbit IgG coupled to Alexa-405 (1:50, Molecular Probes, Inc. Carlsbad, CA) and sheep anti–EGFP primary antibodies, a donkey raised biotinylated anti-rabbit antibody was used to detect rabbit-SV2B antibodies followed by streptavidin-Alexa-405 (Molecular Probes, Inc.). Other secondary antibodies were used as described in Aim 1.

Quadruple color images were obtained in an Olympus FV1000 confocal microscope using laser excitation lines at 405 nm, 488 nm, 568 nm and 633 nm. A
sequential imaging mode was used to minimize cross-talk between detection channels. Renshaw cells were identified according to their ventral positions and high calbindin-IR. Series of confocal optical images (0.5 µm z-steps) throughout calbindin-IR Renshaw cells in ventral LVII and LIX, also labeled for VACHT, EGFP and SV2B were taken at high magnification (60x2.5, NA 1.4). Images were analyzed using Fluoview software. First, all VACHT varicosities in contact with calbindin-IR dendrites were counted and their EGFP content determined by drawing a line through the center of each varicosity and assessing the existence of overlapping, immunofluorescence peaks in the VACHT and EGFP-IR imaging channels. This was then followed in a reverse manner by first counting all EGFP varicosities confirmed as being synaptic boutons by the presence of SV2B-IR and using a similar line analysis to determine colocalization with VACHT-IR.
Results

Motoneurons are the only spinal neurons that co-express EGFP and cholinergic markers (ChAT, VACHT)

EGFP immunofluorescence in spinal cord sections of Hb9::EGFP mice was present in LIX motoneurons, as well as in many LVII interneurons as previously reported (Wichterle et al., 2002; Hinckley et al., 2005; Wilson et al., 2005). Labeling intensity varied between neurons, usually filling the neuron somata, dendrites and axons (Fig. 7). Motor axon EGFP labeling was apparent in the axon-hillock/initial segment region, in the ventral roots, as well as in many axonal-like processes in the Renshaw cell area. However some of these processes could originate from EGFP-IR interneurons. In fact we found that in spinal cord sections through the lower lumbar 4 and 5 segments the number of interneurons labeled was similar to the number of motoneurons. The average number of EGFP-IR motoneurons per ventral horn counted in 6 sections was $33.2 \pm 3.6$ (±SEM) motoneurons per section and the average number of EGFP-IR interneurons was $31.2 \pm 2.7$ (t-test, p=0.665). The presence of EGFP in interneurons of Hb9::EGFP mutant mice has been recognized before (Hinckley et al., 2005; Wilson et al., 2005) and most have been considered to result from ectopic expression of the Hb9::EGFP transgene in non-Hb9 expressing cells. Only a few cells with intense EGFP expression truly express the Hb9 transcription factor. These cells are more brightly labeled, located medially in the ventral horn and are most abundant in the rostral lumbar segments (Hinckley et al., 2005; Wilson et al., 2005). We found few cells with these characteristics in lower L4-L5 segments that however seem to contain more EGFP labeled interneurons overall. Many EGFP interneurons display rather weak immunofluorescence and a larger number might
have been revealed in this study because in all the materials analyzed the EGFP fluorescence was amplified with immunofluorescence. Since the purpose of this study was to test whether motoneurons are the only cholinergic neurons that express the Hb9::EGFP transgene we did not attempt to describe the nature of EGFP-labeled interneurons and their axons.

ChAT-IR was present in motoneurons and certain classes of interneurons (Fig. 7B) in agreement with the distributions previously reported (Barber et al., 1984; Phelps et al., 1984; Miles et al., 2007). However, ChAT and EGFP colocalization was only observed in lamina IX (LIX) neurons, never in interneurons or outside of LIX. ChAT-IR interneurons did not express the Hb9::EGFP transgene at any age (arrowhead, Fig. 7B, C). The distribution plots of ChAT and EGFP labeled cells (Fig. 8A-C) showed that ~92% of LIX ChAT-IR motoneurons expressed EGFP at P0. In older animals there was a progressive decrease in the number of LIX ChAT-IR motoneurons labeled with EGFP (Fig. 8D, F). At P20, the last age analyzed, only ~53% of ChAT-IR neurons in LIX expressed EGFP detectable with immunofluorescence.

VACHT-IR is also present in the somata of motoneurons and a few interneurons, but cell body labeling was less intense than ChAT-IR. This is likely due to the efficient transport of VACHT-IR vesicles to synaptic terminals (Erickson, et al., 1994; Schäfer et al., 1994). Therefore, to examine VACHT-IR in EGFP neurons the antibody NeuN was used to best label the somas (Fig. 9). NeuN is a neuron specific nuclear protein and is highly expressed in α-motoneurons (very recent studies suggest that it is not expressed by γ-motoneurons: Friese et al., 2009, Shneider and Alvarez, submitted). After plotting NeuN/VACHT motoneurons and NeuN interneurons using the Neurolucida system as
described above it was determined that similar to ChAT-IR most P10 large NeuN/VACHT labeled neurons in LIX expressed EGFP. Interestingly, despite the abundance of EGFP labeled interneurons they represent only a small percentage (∼10%) of the total population of ventral interneurons (the ventral horn area was defined as the spinal gray matter below a horizontal line drawn above the dorsal tip of the central canal). None of the NeuN/EGFP-IR interneurons contained VACHT-IR. Correspondingly, VACHT-IR C-terminals on motoneurons, which are of interneuronal origin, did not contain EGFP-IR (Fig. 7I). In contrast, most VACHT-IR varicosities in the Renshaw cell area, which are likely originated from motoneurons, were EGFP-IR (Figs. 10, 11).

**Motor axon synapses on Renshaw cells co-express VACHT and EGFP, but EGFP is down-regulated with development**

EGFP-IR seems to colocalize with cholinergic markers (ChAT and VACHT) exclusively in motoneurons, therefore we next investigated if EGFP and VACHT always co-labeled synaptic varicosities contacting Renshaw cells. If this was the case we could argue that VACHT and/or EGFP labeled synapses on Renshaw cells are always originating from motor axons. EGFP labeling was distributed throughout the axon collateral including the preterminal and intervaricose regions. Thus, EGFP-IR presynaptic varicosities on calbindin-IR Renshaw cells were identified by a high content of SV2B-IR (Fig. 10, 11). Their VACHT-IR content was then tested using line profiles as explained in the methods section and figure 10. All EGFP/SV2B-IR presynaptic varicosities in contact with calbindin-IR Renshaw cells of any age contained VACHT-IR (sample sizes: P0, n=50; boutons P5, n=25; P10, n=50; P20, n=67).
Then we analyzed if there was a large population of VAChT-IR contacts on Renshaw cells that did not contain EGFP (Fig. 11). At age P0 ~91% of VAChT-IR contacts on Renshaw cell contained EGFP-IR and this percentage decreased gradually at P5 and P10 until age P20 in which ~77% of VAChT-IR contacts on Renshaw cells contained detectable EGFP-IR (sample sizes: P0, n=55 VAChT-IR contacts; P5, n=55; P10, n=74; P20, n=100). This decrease parallels the diminished expression of EGFP in motoneurons during development noted above, and therefore the simplest explanation is that the majority of VAChT-IR contacts on Renshaw cells are of motoneuron origin (as shown at P0 when EGFP expression is high). However, with development EGFP content drops below detection level in about 25% of the synapses. It is interesting that down regulation occurs faster on the proximal dendrites and somata than at the distal ends of the axon arborization. Whether or not this reflects a slower turnover of EGFP protein in the axon versus the soma and dendritic region is unknown.

Finally we investigated if other interneurons were targeted by axons with EGFP or VAChT dual immunoreactivity. Renshaw cells and Ia inhibitory interneurons (IaINs) are closely related embryologically (Alvarez et al., 2005), but in the postnatal spinal cord they are differentially targeted by motor axons. Thus, mature IaINs are expected to lack a significant motor axon input. A group of IaINs in the ventral horn can be characterized by their parvalbumin-IR and extensive proximal inputs from calbindin-IR Renshaw cell boutons (Alvarez et al., 2005). Parvalbumin-IR IaINs received contacts from VAChT-IR boutons (Fig. 12), but of the 138 VAChT-IR contacts counted on sixteen P15 parvalbumin-IR interneurons only 3 (~2%) contained EGFP-IR. Therefore most VAChT inputs on IaINs likely arise from sources other than motoneurons.
Figure 7: Colocalization of EGFP and ChAT or VACHT immunoreactivity in P10 Hb9::EGFP mice. A-C) Confocal microscopy images of a ventral horn dual immunolabeled for EGFP (A, FITC, green), and ChAT (B, Cy3, red). C, shows colocalization. EGFP labels motoneurons in LIX (large size neurons) and many interneurons (smaller sized cell bodies) throughout LVII. ChAT-IR labels mostly motoneurons and a few interneurons in medial locations close to the central canal (arrows in B and C). EGFP and ChAT were co-localized in LIX motoneurons, but not in interneurons. D-F) Confocal images of a ventral horn dual immunolabeled for EGFP (D) and VACHT (E). F, shows colocalization. EGFP-IR labels motoneurons and interneurons as before. VACHT is co-expressed in ChAT-IR neurons, however the protein is mostly trafficked to the synaptic boutons (punctae in the image) and VACHT-IR levels are lower in cell bodies compared to ChAT-IR. Nevertheless, both motoneurons (in lamina IX, LIX) and interneurons (arrows in E and F) in dorsomedial lamina VII, LVII) can be identified. As before, VACHT-IR colocalized with EGFP in motoneuron cell bodies, but not in interneurons. G-I) Motoneurons (green) express EGFP in Hb9::EGFP mice in their somas as well as all their processes. VACHT-IR (red) C-terminals surround and contact motoneuron cell bodies and proximal dendrites. These terminals are from an interneuronal source and lack EGFP labeling. Scale bar in F is 100µm; 20µm in I.
Figure 8: Quantitative analysis of EGFP and ChAT in Hb9::EGFP mice. A-C) Neurolucida plots of ChAT-IR motoneurons (black triangles) and interneurons (open triangles) and colocalization with EGFP-IR motoneurons (open circles) or lack of colocalization with EGFP-IR interneurons (black circles). Data plots are from a single 50 µm thick sections of a P10 Hb9::EGFP mouse. D-E) Fewer ChAT-IR motoneurons (Cy3, red) show EGFP-IR (FITC, green) at P20 (E) than at P0 (D). F) The percentage of ChAT-IR motoneurons expressing EGFP progressively decreases from P0 to P20. Data was obtained from ChAT-IR motoneurons in 6 ventral horn sections at each age (P0=231 motoneurons, P5=182 motoneurons, P10=161 motoneurons, P20=138 motoneurons). Error bars indicate S.E.M. from the 6 sections. One animal was analyzed at each age. Scale bars in D and E are 100µm.
A  ChAT-IR motoneuron
△ ChAT-IR interneuron
○ EGFP-IR motoneuron
● EGFP-IR interneuron

% of ChAT MNs expressing EGFP

- P0
- P5
- P10
- P20
Figure 9: EGFP expression in VACHT-IR motoneurons and NeuN-IR interneurons.

A) NeuN (Cy3, red), a neuronal cell marker labels motoneurons in lamina IX (LIX) and interneurons in lamina VII (LVII) and throughout the spinal cord. B) EGFP-IR (FITC, green) is found in most of the large NeuN labeled neurons in LIX and also in a portion of NeuN-IR interneurons in LVII. C) VACHT-IR (blue) weakly labels motoneuron somas in LIX, but is not found in any of the EGFP-IR interneurons in LVII. D) At postnatal day 10 (P10), almost all (96.2%) large NeuN labeled neurons within LIX (presumable motoneurons) contain EGFP-IR. In contrast, only a relatively small percentage (10.1%) of NeuN-IR interneurons in the ventral horn contains EGFP-IR. E) Most of the large NeuN-IR and VACHT-IR motoneurons contain EGFP-IR (91.6%), whereas less than 1% of ventral horn interneurons contain VACHT- and EGFP-IR. Scale bar in C equals 50 μm. A and B panels are at the same magnification.
Figure D shows the percent of large and small NeuN neurons labeled with EGFP. The bar graph indicates that 96.2% of large NeuN neurons and 10.1% of small NeuN neurons are labeled with EGFP.

Figure E illustrates the percent colocalization of large NeuN neurons with EGFP and VACHT. The bar graph reveals that 91.6% of large NeuN neurons with EGFP and 0.24% of small NeuN neurons with EGFP and VACHT are colocalized.
Figure 10: Colocalization of EGFP-IR and VACHT-IR in synaptic contacts on calbindin-IR Renshaw cells. A-D) High magnification confocal images of a calbindin-IR Renshaw cell at age P10 (Cy5, blue). This Renshaw cell is contacted by some EGFP-IR varicosities (FITC, green; yellow arrow in A). B) SV2B-IR (Alexa 405, white) is superimposed to show its occurrence inside some of EGFP-IR varicosities contacting Renshaw cells. This suggests the presence of synaptic vesicles within the EGFP-IR contact varicosity. C) VACHT-IR (Cy3, red) boutons contacting the Renshaw cell. D) VACHT-IR colocalization in EGFP/SV2B-IR synaptic contacts on calbindin-IR Renshaw cells. Varicosities 1 and 2 were analyzed in the line profiles shown in E and F. E) Line analysis of synapse 1 shows corresponding fluorescence intensity peaks along the line for the VACHT, EGFP and SV2B immunoreactivities. F) Line analysis of synapse 2 (not contacting the Renshaw cell) shows corresponding VACHT and SV2B immunofluorescence peaks but lack of EGFP content. Scale bar in D equals 10µm. All panels at the same magnification.
Figure 11: EGFP inside VACHT-IR synapses on Renshaw cells decreases with development. A-D) High magnification confocal images of a P10 Renshaw cell. A) The calbindin-IR Renshaw cell (Cy5, blue) receives contacts from many EGFP-IR axonal varicosities (FITC, green). B) SV2B-IR (Alexa 405, white) is contained at many of this EGFP-IR contacts confirming their presynaptic nature. C) VACHT-IR boutons (Cy3, red) contact the Renshaw cell. D) VACHT-IR is contained in many EGFP-IR/SV2B-IR synaptic contacts on Renshaw cells. All EGFP-IR synapses on calbindin-IR Renshaw cells contain VACHT-IR. E-H) Similar high magnification confocal image of a Renshaw cell at age P20 labeled as before with EGFP, SV2B and VACHT. The amount of EGFP-IR contacts on Renshaw cell decreases in P20 mice compared to P10 mice. Nonetheless, many of the contacts contained SV2B-IR indicating that they are synapses. All of them contained VACHT-IR. However, at this age a significant proportion of VACHT-IR contacts on Renshaw cells lacked EGFP-IR. I) Percentage of VACHT-IR boutons in contacts with calbindin-IR Renshaw cells that contain EGFP-IR in line analyses illustrated in Figure 10. At P0 ~91% of VACHT-IR contacts on calbindin-IR Renshaw cells also contain EGFP-IR. However, as the animal ages the percentage of VACHT-IR containing EGFP-IR decreases to ~77%. J) Proportion of EGFP-IR/SV2B contacts on Renshaw cells. At all ages examined all EGFP-IR/SV2B contacts contained VACHT-IR. Scale bar in H equals 20 µm. All other confocal images are at the same magnification.
**Figure 12**: Ia inhibitory interneurons do not receive cholinergic input from motoneurons. Analysis was done in P15 spinal cords. A) Low magnification image of EGFP-IR (FITC, green), VACht-IR (Cy5, white), parvalbumin-IR (Cy3, red; see arrow for example) and calbindin-IR (Alexa-405 blue; see arrowhead for example of a calbindin-IR Renshaw cell) in the spinal cord ventral horn. B-D) High magnification images of a parvalbumin-IR IaIN (see arrow in A) receiving contacts from VACht-IR boutons (B). This parvalbumin-IR IaIN receives many contacts from calbindin-IR Renshaw cells (blue) as well as from EGFP-IR axons (green) (C). However VACht-IR contacts on parvalbumin-IR IaINs lacked EGFP-IR. E-G) Calbindin-IR Renshaw cells (blue) receive VACht-IR contacts (white in E) that frequently are EGFP-IR (green/white colocalization in G). G) Average percentages of VACht-IR contacts containing EGFP-IR on P15 calbindin-IR Renshaw cells and parvalbumin-IR IaINs (error bars indicate S.E.M.s). Most VACht-IR contacts on Renshaw cells contain EGFP. Almost none of the VACht-IR contacts on parvalbumin-IR IaINs contain EGFP indicating that EGFP-expressing motoneurons are the source of VACht-IR contacts on calbindin-IR Renshaw cells, but not on parvalbumin-IR IaINs. Scale bar in A equals 100 µm. Scale bar in G equals 10 µm. Images in B-G are at the same magnification.
Discussion

The results suggest that the only cholinergic neurons in the spinal cord that expresses EGFP in Hb9::EGFP mice are motoneurons. Thus, dual localization of EGFP/ChAT-IR or EGFP/VAChT-IR specifically identifies motoneuron somata, dendrites, and axons. Using these criteria we concluded that practically all EGFP-IR or VAChT-IR synaptic boutons on Renshaw cells have a motoneuron origin. In neonatal animals most VAChT-IR contacts on Renshaw cell contain EGFP and vice versa. This result rules out the possibility that Renshaw cells receive significant projections from EGFP-expressing interneurons or the existence of significant populations of motor axons lacking VACHT. In contrast, VAChT-IR boutons contacting non-Renshaw cells, like IaINs and motoneurons, did not contain EGFP-IR. VAChT-IR terminals on motoneurons are known as C-terminals and are thought to originate from a pool of cholinergic interneurons, namely medial partition neurons (Miles et al., 2007) located dorsomedially in the ventral horn. The Neurolucida analysis confirmed that cholinergic interneurons do not express EGFP and accordingly C-terminals were also EGFP-negative. The source of VAChT-IR contacts on IaINs remains unknown.

In Hb9::EGFP mice EGFP expression in motoneurons and interneurons decreased with age and because of this an increased portion of VAChT-IR boutons did not contain EGFP in older animals. No evidence was found at any age for a significant population of EGFP varicosities presynaptic to Renshaw cells and lacking VACHT. As Hb9 is an important transcription factor in the development of motoneurons (Arber et al., 1999; Wichterle et al., 2002) it is not surprising that the amount of EGFP expressed decreases postnataally. An alternative reason for the decrease in EGFP seen in the older animals is
that as the animal ages the motoneurons increase in size and this might lead to a dilution of EGFP that becomes undetectable using standard immunocytochemistry.

Overall, this analysis validated the use of EGFP-IR or VACHT-IR as markers of central motoneuron synapses contacting Renshaw cells. EGFP-IR and VACHT-IR are internal markers and therefore \textit{in vitro} tracing techniques are not required to label motoneuron processes. Optimal fixations by transcardial perfusion for immunolabeling of excitatory amino acids are then attainable. However, the use of EGFP-IR as a marker for motor axon contacts on Renshaw cells could be problematic because decreased labeling with age restricts the studies to neonatal animals. Moreover, EGFP-IR is not only present in motor axons but also intensely labels dendrites throughout LIX and the Renshaw cell area of LVII. This makes it difficult to efficiently detect motor axons in the neuropil. Our preliminary electron microscopy work revealed the complexity of detecting EGFP-IR motor axon processes within a dense plexus of more heavily immunolabeled dendrites. As a result few examples of EGFP-IR synapses on Renshaw cells were identified after much effort.

To try to restrict immunolabeling to the motor axons we attempted crossing Hb9\textsuperscript{cre/+} mice with two different reporter mice: R26\textsuperscript{GAP43-EGFP/+} mice (Sapir et al., 2004; Zhang et al., 2008) and Tau\textsuperscript{mEGFP-nlslacZ/+} mice (Hippenmeyer et al., 2005). Using these reporter lines we tried to direct EGFP labeling specifically to the motor axons either through the GAP43 axon trafficking signal in the construct expressed in R26\textsuperscript{GAP43-EGFP/+} mice or by the myristilation site of mEGFP expressed in Tau\textsuperscript{mEGFP-nlslacZ/+} mice. However, two attempted crossings with each of these lines of mice failed to produce the expected labeling outcome of localizing EGFP to motor axons only. Labeling was
observed in neurons throughout the spinal cord as well as in glial-like structures. The reasons for this lack of specificity in genetic-labeling are presently unknown, but suggest an unexpected widespread expression of Cre recombinase in Hb9Cre/+ mice that contrast with recent studies that assumed specific expression in motoneurons (see for example Zhang et al., 2008). The failure of these crossings to produce clear motor axon labeling led us to conclude that VACHT antibodies are the best marker available to us for identification of motor axon synapses on Renshaw cells in the electron microscopy experiments of Aim 3. As made clear by this Aim, VACHT-IR at synapses contacting Renshaw cells is a specific indicator of motor axon terminals. This is in agreement with previous observations that VACHT-IR boutons contacting Renshaw cells can be retrogradely labeled from motor axons (Aim 1 and Mentis et al., 2005) and that they degenerate in mature SOD1-mice undergoing a motor axon pathology characteristic of amyotrophic lateral sclerosis (Fitzsimons et al., 2006). However in these previous studies it could not be definitively proven whether the majority or only a portion of VACHT-IR contacts on calbindin-IR Renshaw cell dendrites were of motor axon origin. The use of a genetically encoded label in this aim demonstrated that almost all, if not all, VACHT-IR boutons in contact with Renshaw cell indeed originate from motor axons.
VI. Aim 3: Electron microscopy analyses of aspartate and glutamate enrichment in motoneuron synapses on Renshaw cells

Rationale:

The results shown in Aim 1 suggest that VGLUT-IR is undetectable at central motoneuron synapses. This result raises questions about whether glutamate, or another excitatory amino acid, like aspartate is accumulated in the synaptic vesicles of motoneurons. VGLUTs are specific transporters for glutamate and are unable to transport aspartate into synaptic vesicles (Bellocchio et al., 2000; Herzog et al., 2001; Fremeau et al., 2002, Varoqui et al., 2002). One possible explanation for the contrasting electrophysiological (i.e. activation of AMPA and NMDA receptors) and immunocytochemical results (lack of VGLUTs) is that aspartate instead of glutamate is released by motor axon terminals on Renshaw cells. Therefore it is important to test directly the presence of glutamate and/or aspartate in motor axon synapses. However, glutamate and aspartate are generated during normal metabolism and are ubiquitously present in motoneurons and other cell types. To show a possible role in neurotransmission it is necessary to specifically demonstrate enrichment of these excitatory amino acids in the vesicle pools of these synaptic terminals. Towards this aim the immunoreactivities for aspartate and glutamate in motor axon terminals were quantified using electron microscopy immunocytochemistry. Two issues have to be first considered before proceeding with quantification of electron microscopy immunocytochemistry experiments: 1) A method for motor axon synapse identification at the ultrastructural level and 2) The specificity of antibodies against small amino acids of closely related structure.
From Aim 2 it was confirmed that VACHT is a reliable marker of motoneuron contacts on Renshaw cells. Therefore we used pre-embedding electron microscopy techniques to label calbindin-IR Renshaw cells and VACHT-IR motoneuron terminals contacting Renshaw cells. This was then combined with post-embedding quantitative colloidal gold immunostaining to determine if either aspartate or glutamate-IR is enriched in vesicular pools.

Regarding specificity, the structure of glutamate is very similar to aspartate, differing only by a single methyl group. Therefore the likelihood of cross reactivity using polyclonal antibodies is very high. It is important to thoroughly test the specificity of antibodies for their targeted amino acid neurotransmitters. For this purpose we used the sandwich method published by Ottersen (1987). This method is described in detail in the Methods section.
Materials and methods

Antibody specificity

The specificities of the aspartate (rabbit, polyclonal) (Biogenesis Ltd., Poole, UK) and glutamate (rabbit, polyclonal) antibodies (Alpha Diagnostics International, Inc., San Antonio, TX, and Biogenesis Ltd., Poole, UK) were tested using resin sandwiches kindly provided by Dr. O. P. Ottersen (University of Oslo, Norway). In this procedure, different amino acids were conjugated to brain homogenates with glutaraldehyde and then embedded in Durcupan resin. The production of these test sandwiches is fully described elsewhere (Ottersen, 1987). Briefly, 2 µm semi-thin sections were obtained from each Durcupan-embedded amino acid enriched brain homogenate and from a brain homogenate similarly processed but not conjugated to any amino acids (blank control). These sections were stacked between semithin sections of fixed and resin-embedded brain tissue. These stacks of sections were then re-embedded in resin and transverse ultrathin sections were cut perpendicular to the entire stack rendering ultrathin sections containing layers of resin with islands of amino acids conjugated to brain homogenates sandwiched between layers of brain tissue. Dr. Ottersen’s lab kindly donated a few grids containing ultrathin sections of these amino acid embedded resin sandwiches to test our antibodies. The layers of brain homogenate “islands” contained the following glutaraldehyde-fixed amino acids: aspartate, GABA, glutamate, glutamine, glycine, taurine, and a homogenate layer containing no amino acids. Electron microscopy grids containing these test sections were rinsed in Tris buffer saline of pH 7.6 containing 0.1% Tween (TBS-Tw) and were then incubated for 30 minutes in 10% normal goat serum (Vector) and then overnight at 4°C in a primary antibody solution containing either rabbit
anti-glutamate (0.17 µg/ml & 0.034 µg/ml) or rabbit anti-aspartate (0.36 µg/ml and 0.18 µg/ml) in TBS-Tw pH 7.6. Following primary antibody incubations the grids were rinsed in TBS-Tw pH 7.6 followed by a rinse in TBS-Tw pH 8.2. After rinsing, the grids were incubated in a secondary antibody solution containing anti-rabbit antibodies conjugated to 10 nm colloidal gold particles (British BioCell International (BBI), Cardiff, UK) at 1:25 dilution in TBS-Tw pH 8.2 for 2 hours. Following this incubation, grids were rinsed in nanopure distilled water (ndH₂O). The ultrathin sections were then contrasted using a 1% uranyl acetate solution followed by rinses in ndH₂O, followed by incubation in lead citrate and more rinses in ndH₂O. High magnification images (100,000x) were taken of the amino acid enriched brain homogenate “islands” with a digital camera coupled to the electron microscope (Gatan, Bio Scan II, Pleasanton, CA). The cross-sectional area of each “island” was traced and the number of gold particles found on top divided by the area of the “island” to determine the density of gold particles.

**Pre-embedding electron microscopy immunolabeling for calbindin and VACHT**

Three P20 mice were anesthetized with pentobarbital (Nembutal or Euthasol 50-70 mg/kg i.p.) and perfused transcardially, first with a cold vascular rinse (0.01M phosphate buffer with 137mM NaCl, 3.4mM KCl, and 6mM NaHCO₃, pH 7.3) followed by fixative containing 2.5% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA) and 1.0% paraformaldehyde (Fisher Scientific) in 0.1M phosphate buffer. The spinal cords were then removed and post-fixed in the same fixative for 4 hours. The spinal cord tissue was stored in 0.01M phosphate buffered saline (PBS) prior to being cut into 100 µm thick vibratome sections. The sections were treated with 1% NaBH₄ (Sigma, Aldrich)
in 0.01M PBS for 20 minutes to remove excess of glutaraldehyde fixation. After rinsing the tissue in 0.01M PBS it was blocked for 30 minutes in 10% normal donkey serum (Jackson ImmunoResearch) diluted in 0.01M PBS. The tissue was then incubated in goat anti-VACHT polyclonal antibodies (Millipore, Temecula, CA) diluted 1:1,000 in 0.01M PBS overnight on an orbital shaker at 4°C. To reveal VACHT-IR we used a silver amplified nanogold method. Sections were rinsed in 0.01M PBS and incubated in a biotinylated-SP anti-goat secondary antibody (Jackson Immunoresearch) diluted 1:100 in 0.01M PBS. Following this incubation the tissue was rinsed in 0.01M PBS and then incubated in streptavidin coupled to 1.4 nm gold particles (Nanoprobes, Yaphank, NY) at a 1:50 dilution in 0.01M PBS. The tissue was again rinsed in 0.01M PBS and then in 50 mM glycine in 0.01M PBS. Bovine serum albumin at 1.0% (Sigma-Aldrich) in 0.01M PBS was used to reduce background labeling. The tissue was then rinsed briefly in ndH2O before being silver intensified with an HQ Silver Enhancement Kit (Nanoprobes) to reveal VACHT IR boutons. The tissue was placed in the silver solution for 7-11 minutes and then rinsed multiple times in ndH2O, then in 0.01M PBS, and then in 0.2M Tris maleate buffer. The silver reaction product was stabilized with 0.05% gold chloride solution followed by rinses in 0.2M Tris maleate buffer, a brief incubation in 3% sodium thiosulfate solution to dissolve any remaining unbound silver, and then rinsed in 0.02M Tris maleate buffer and in 0.01M PBS. Following these rinses the tissue sections were again incubated overnight in a new primary antibody solution containing rabbit anti-calbindin (Swant), diluted 1:500 in 0.01M PBS to label Renshaw cells and their processes. To reveal the calbindin-IR we used ABC-peroxidase and standard Vector kits (Vector Labs). Peroxidase-labeled immunoreactive sites were revealed with 0.02%
diaminobenzidine (DAB) and 0.01% hydrogen peroxide with the reaction time lasting between 5 and 8 minutes. Finally, the tissue was rinsed in 0.01M PBS.

**Cryosubstitution and Lowicryl embedding**

The immunolabeled sections were cryoprotected in an ascending series of glycerol solutions diluted in 0.1M PB (30 minute incubations in solutions at 10%, 20%, and 30% strength), followed by overnight incubation in 30% glycerol. The sections were then freeze-slammed using a “copper-mirror” technique in a Leica MM80 plunger. Following freeze slamming the tissue was placed at -80°C in a cryosubstitution chamber and rinsed 4 times in methanol for 30 minutes each. This was followed by a 1:1 methanol:Lowicryl (Electron Microscopy Sciences, (EMS) Hatfield, PA) incubation for 2 hours at -50°C, a 1:2 methanol:Lowicryl incubation for 2 hours at -50°C, a 100% Lowicryl incubation for 2 hours at -50°C, and a fresh 100% Lowicryl incubation overnight at -50°C. The Lowicryl tissue was then flat-embedded between teflon-coated glass coverslips and placed under ultraviolet light using the following temperature and schedule for resin polymerization: -50°C for 49 hours, raised to 0°C over a time of 5 hours, held at 0°C for 72 hours, raised to 20°C over a time of 10 hours, followed by a 24 hour incubation at 20°C.

**Colloidal gold post-embedding with aspartate and glutamate antibodies**

Areas of interest containing calbindin-IR Renshaw cells were identified under the light microscope in flat embedded section wafers, re-cut and glued at the top of pre-polymerized EM chucks. Ultrathin sections (~70-90 nm thick, silver or silver-gold
diffraction color) were obtained and collected on 200 mesh nickel grids coated with Formvar (EMS). The grids were then processed for postembedding colloidal gold immunostaining in the same manner and with the same dilutions of aspartate and glutamate antibodies as described above for testing of the antibody specificity on resin sandwich grids. The sections were contrasted with 1% uranyl acetate and lead citrate.

**Electron microscopy image capturing and analysis**

Contrasted and immunolabeled ultrathin sections were examined in a Phillips 208S electron microscope at 70 kV (Aachen, Germany). Images were obtained photographically (EM 4x5 negatives) and the negatives scanned at 1200 dpi (Flextight 848; Imacom, Copenhagen, Denmark). Micrographs were obtained from at least 2 grids containing 2 to 4 ultrathin sections per each of the 3 mice. Scanned images containing VAChT-IR boutons synapsing on either Renshaw cells or motoneurons were analyzed using Image Pro Plus (version 5.1; Media Cybernetics, Silver Spring, MD). The area of axoplasm from which neurotransmitters could be loaded into synaptic vesicles was determined by measuring the area of the bouton and subtracting the area occupied by mitochondria. Then the number of colloidal gold particles inside the bouton, but outside mitochondria, was counted and a density calculated (number of gold particles per 1 µm²). Average densities of gold particles were estimated for each animal or the whole sample for VAChT-IR boutons contacting DAB labeled Renshaw cells dendrites (motor axons) and VAChT-IR boutons contacting motoneurons (mostly C-terminals). To determine if there was immunogold enrichment inside VAChT-IR boutons above average neuropil staining, 1 µm² samples of neuropil were similarly analyzed. Neuropil was collected from
the regions at the four corners of the scanned negatives. Similar to the boutons, mitochondria areas and gold particles were subtracted from the density calculation. Any neuropil test squares covering areas of the photograph that contained VACHT-IR synapses, or artifacts like precipitates, holes, or grid bars passing through the image were excluded.

**Statistical comparisons**

Statistics were always used to compare groups of data for differences in immunolabeling density. When possible paired groups of data were compared using standard t-tests and multiple groups (more than two) with standard one-way ANOVAS followed by pair-wise comparisons among the groups using a Tukey test. However data were frequently not normally distributed. This was usually the case in samples with very low levels of immunolabeling and few skewed data points or in samples with several estimates at zero. In these cases we used non-parametric tests: Mann-Whitney Rank-Sum test for comparing two groups and Kruskal-Wallis ANOVA on Ranks for more than two groups followed by post-hoc Dunn’s test for pair-wise comparisons. Statistical significance was always set at p<0.05. All statistical comparisons were run in Sigma-Stat 3.1 (Jandel Corporation, Chicago, IL).

To estimate the percentage of boutons positively labeled we established a criterion for labeling threshold at a density two standard deviations above average neuropil labeling. Percentages were obtained for the number of boutons displaying labeling above this density.
Results

Specificity of polyclonal aspartate and glutamate antibodies

Specificity was determined on layers of brain homogenates containing various glutaraldehyde-fixed amino acids (aspartate, GABA, glutamate, glycine and taurine) or no amino acid. Brain homogenate particles (“islands”) enriched with the different amino acids were sandwiched in between layers of brain tissue (Fig. 13). Following aspartate and glutamate post-embedding immunohistochemistry, we determined the density of 10 nm colloidal gold particles over the different amino acid enriched brain homogenates (Figs. 14 and 15). Two different antibody concentrations were used in each test. Ten islands enriched for each amino acid were imaged and analyzed in each immunolabeling.

At both antibody dilutions, aspartate immunolabeling was higher in aspartate islands compared to other amino acids (Fig. 14A,B) and the differences in average density were always significant (p<0.001, Kruskal-Wallis ANOVA on Ranks, p<0.05 Post-hoc Dunn’s test). Aspartate immunolabeling density in individual aspartate enriched islands ranged from dense to weak and was rather similar at both antibody dilutions, except for few very weakly labeled islands using the weaker antibody concentration (Fig. 15A,B). However average labeling density in aspartate islands was not significantly different between antibody dilutions (t-test, p=0.756). The major difference noted was that at the more concentrated antibody dilution a small proportion of islands enriched with other amino acids showed some, albeit quite variable, immunolabeling. In particular a few glutamate enriched islands showed significant immunolabeling with concentrated aspartate antibodies, but as a group glutamate enriched islands were not statistically
different from control blank brain homogenates (p=0.426, Mann-Whitney Rank Sum test).

Glutamate immunolabeling was significantly higher on glutamate enriched islands when using the more concentrated antibody dilution (0.17 µg/ml) compared to the least concentrated (0.034 µg/ml) (t-test, p<0.001) (Fig. 15C, D). Glutamate immunolabeling at both dilutions was always significantly higher on glutamate enriched islands compared to any other amino acid enriched islands or controls (p<0.001, Kruskal-Wallis ANOVA on Ranks, p<0.05 Pot-hoc Dunn’s test). With the exception of glutamine, all other amino acid enriched islands were no different from control (blank). Glutamate-immunolabeling of glutamine islands was significantly higher from controls at both dilutions (p<0.01, Mann-Whitney Rank Sum test).

In conclusion, the aspartate antibodies appear quite specific for glutaraldehyde-fixed aspartate, but at high concentration might sometimes weakly recognize glutamate. Glutamate antibodies in contrast do not recognize aspartate at the dilutions used, but they have significant cross-reactivity with glutamine.

VACHT-IR and calbindin-IR structures in the ventral horn Renshaw cell area

Silver intensified nanogold VACHT immunoreactivity is recognized as relatively large and irregularly shaped dark silver deposits. These deposits were found in cholinergic synapses contacting Renshaw cells and at a lower density in motoneuron somata and dendrites, as well as at very high density in some cholinergic terminals on motoneuron somata known as C-terminals (Fig. 16, Nagy et al., 1993). The relative density of the silver deposits inside boutons was maximal on the vesicle pools but the
absolute density varied depending on the depth at which the ultrathin section was obtained within the thickness of the 50 µm immunolabeled section. Labeling was denser at the surfaces of the section and decreased significantly after the first 5-10 µm deep into the tissue section. This is due to the relatively poor penetration of antibodies, particularly the nanoprobes, in the thick sections during pre-embedding immunostaining. Terminals with very dense VACHT labeling were excluded from quantification (Fig. 16, bouton at the bottom left and labeled with two asterisks), as it was difficult to detect 10 nm colloidal gold particles within a high density of silver deposits. VACHT-IR terminals with weaker labeling (Fig. 16, boutons at the top of the image and labeled with one asterisk) were ideal for quantifying colloidal gold immunolabeling. As mentioned above the intensity of labeling was not related to the total amount of synaptic vesicles (and thus VACHT antigen content), but to the depth in the tissue at which the terminal was sampled.

C-terminals contacting motoneuron somata were characterized by the presence of a subsynaptic cistern (Fig. 16, bottom inset). C-terminals do not originate from motor axons and are therefore different from motor axon synapses on Renshaw cells. Not all VACHT-IR terminals contacting motoneurons had a detectable subsynaptic cistern. Unfortunately in triple immunolabeled and non-osmicated sections ultrastructural preservation is compromised and therefore it was sometimes difficult to conclude whether subcisterns are truly lacking from some of the terminals or just non-resolvable. Therefore, a portion of VACHT-IR synapses on motoneurons may not be C-terminals and could originate from motor axons (Lagerback et al., 1981).
Calbindin-IR Renshaw cell dendritic profiles were labeled with diaminobenzidine (DAB). DAB labeling appears as electron dense diffuse precipitate that is hydrophobic forming fine halos on lipid membranes, such as the plasma membrane or membranes surrounding cell organelles. DAB labeling also diminishes with increasing depth in the section. Moreover, DAB density was generally relatively weak in Lowicryl embedded tissue, as with cryosubstitution techniques, osmium is not used to amplify DAB electron density. Cryosubstituted materials were used for aspartate and glutamate immunoreactions because preliminary experiments demonstrated a higher sensitivity of post-embedding colloidal gold immunolabeling using this method. Calbindin-IR was observed in Renshaw cell somata and dendrites that were contacted by VACHT-IR terminals (Figs. 16, 17, 20, 21A), as well as in Renshaw cell synaptic terminals (Figs. 18, 21B). The postembedding method used to label either aspartate or glutamate is identical to the procedure described above to test antibody specificity in ultrathin sections from amino acid enriched wafers and results in uniformly round 10 nm colloidal gold particles that can be easily distinguished from immunogold-silver and DAB deposits.

**Aspartate immunoreactivity**

Many VACHT-IR terminals making synapses on calbindin-IR Renshaw cells (i.e., motor axon terminals) contained colloidal gold aspartate-immunolabeling above neuropil immunostaining. Aspartate-IR was observed on the axoplasm, over synaptic vesicle pools (Fig. 17A), as well as over organelles including some mitochondria (see arrows, Fig. 17B). Serial sections of the same synapse contained similar amounts of aspartate-IR
(Fig. 17C, D). Renshaw cell synaptic boutons (Fig. 18), which are inhibitory in nature, or the postsynaptic dendrites, did not contain aspartate-IR at levels higher than the neuropil.

To quantify aspartate-IR in VACHT-IR motor axon terminals, the bouton area was outlined and measured and the area occupied by mitochondria subtracted. Colloidal gold particles were counted only outside mitochondria. Subtraction of mitochondrial labeling was intended to focus the analysis to synaptic vesicle pools and axoplasm regions from where synaptic vesicles could directly accumulate aspartate. Synaptic vesicle immunolabeling content could not be directly estimated because indirect colloidal gold immunostaining using 10 nm particles absorbed to IgGs lacks enough resolution to determine whether immunoreactivities are located inside or outside synaptic vesicles of 30 to 50 nm in diameter (immunoreactive sites are estimated to localize within a 30 nm radius of the edge of the gold particle; Matsubara et al., 1996).

Colloidal gold aspartate-immunolabeling was estimated using low (0.18 µg/ml) and high (0.36 µg/ml) antibody concentrations to better resolve possible differences with neuropil labeling. Thirty-four and 25 VACHT-IR motor axon boutons presynaptic to calbindin-IR Renshaw cell dendrites were analyzed respectively at each antibody concentration (Fig. 19A,B). Boutons were sampled from tissue blocks obtained from three different animals. Averaging the data from all boutons sampled in the three animals resulted in a colloidal gold average density inside VACHT-IR motor axon terminals that was significantly higher at 0.36 µg/ml antibody concentration than at 0.18 µg/ml (21.5 particles/µm² ± 1.9 SEM, vs. 13.8 ± 0.2, p=0.005, t-test) (Fig. 19E, F). Similarly, average neuropil labeling was significantly higher at 0.36 µg/ml antibody concentration compared to 0.18 µg/ml (9.3 ± 0.6 vs. 5.5 ± 0.4, p<0.001, t-test). Average aspartate
immunolabeling inside VACHT-IR motor boutons was significantly greater than on the surrounding neuropil at both antibody dilutions within each of the three animals analyzed (p<0.001, ANOVA, post-hoc Tukey test p<0.001) or after combining all boutons and background estimates from all three animals (p<0.001, t-tests at each dilution) (Fig. 19E, F). Aspartate enrichment in VACHT-IR motor terminals with respect to neuropil (labeling in VACHT-IR motor terminals / labeling in neuropil) was not significantly different between both antibody concentrations (2.8X neuropil density ± 0.8 SEM at 0.36 µg/ml and 2.5 ± 0.03 SEM at 0.18 µg/ml; p=0.31, t-test).

Although comparative enrichment in aspartate immunolabeling was similar in all three animals, absolute values of labeling intensity sometimes varied from animal to animal. Inter-animal differences reached significance at the more diluted antibody concentration (p<0.05, ANOVA, Fig. 19D). At this dilution VACHT-IR motor terminals in animal 1 contained significantly less aspartate immunoreactivity than in animal 2 (post-hoc Tukey Test, p<0.05). Labeling densities from animal 3 were in between estimates for animals 1 and 2, and differences were not significant with either. We also noted variability between animals in basal neuropil immunoreactivity. At low antibody concentration (0.18 µg/ml) variability in neuropil labeling between animals (ANOVA, p<0.001) parallel the inter-animal differences observed for aspartate immunolabeling of VACHT-IR motor axon synapses. At 0.36 µg/ml antibody concentration neuropil labeling was significantly higher in animal 1 compared to the other two animals (ANOVA, p<0.001) (Fig. 19C). Inter-animal differences might reflect disparities in the amount of aspartate antigenicity preserved in each animal during fixation and also the intrinsic variability of postembedding colloidal gold labeling. Despite this variability,
VACHT-IR axon terminals were consistently enriched with aspartate immunoreactivity. Pooling together all boutons analyzed in the three animals, 80% and 71% (for the low and high antibody concentrations, respectively) displayed aspartate immunolabeling two standard deviations above the average neuropil labeling.

The density of aspartate-IR colloidal gold particles in VACHT-IR terminals contacting motoneurons (C-terminals) was consistently lower than in VACHT-IR motor axon terminals contacting Renshaw cells in all three animals and at both antibody concentrations (calculated average densities in C-terminals were 9.6 particles/µm² ± 0.8 at high antibody concentration vs. 8.2 ± 0.8 at low concentration; differences with motor axon terminals were always significant, p<0.001 ANOVAs, post-hoc Tukey test p<0.001) (Fig. 19E, F). Differences between C-terminals and neuropil immunolabeling were generally low but reached significance at the lower antibody concentration (8.2 ± 0.8 vs. 5.5 ± 0.4; p<0.001, t-test) (Fig. 19F). This difference was however inconsistent among samples obtained from different animals (Fig. 19D, t-test p=0.008 for animal 1; p=0.06 for animal 2 and p=0.178 for animal 3). Only a minority of VACHT-IR C-terminals, 17% for the low antibody concentration and 11% for the high, contained aspartate-IR density two standard deviations above their respective animal average neuropil labeling. C-terminals with aspartate labeling at this level were frequently outliers (Fig. 19A, B). Because the unavoidable ultrastructural deterioration that occurs in these complex immunolabeling experiments subsynaptic cisternae were not always unambiguously identified. Thus, we cannot exclude the possibility that a few VACHT-IR contacts on motoneurons could be originated in aspartate-enriched motor axons. Nevertheless the
data do allow us to conclude that C-terminals, in general, do not contain aspartate immunolabeling at densities significantly above the general levels found in the neuropil.

In summary, aspartate immunolabeling seems to be specifically enriched in the axoplasm of cholinergic synapses originating from motor axons and making synaptic contact with Renshaw cells.

**Glutamate-immunoreactivity**

Glutamate-IR densities were analyzed in the same manner, also using two antibody dilutions (0.17 µg/ml or 0.034 µg/ml) and in tissue sections from the same three animals. Immunolabeling above neuropil levels was noted in a portion of VACHT-IR synapses on calbindin-IR Renshaw cell dendrites and was more evident at the concentrated antibody dilution (Fig. 20). Glutamate-IR enrichment was also frequently observed on vesicle pools of neighboring unlabeled Type I excitatory synapses. Inside these synapses glutamate-IR appeared more intense than in VACHT-IR synapses (Fig. 21A, bouton labeled with an asterisk). Renshaw cell inhibitory synapses labeled with DAB did not contain glutamate-IR colloidal gold above neuropil levels (Fig. 21B). In contrast to aspartate-IR, C-terminals frequently contained glutamate-IR clearly above neuropil levels (Fig. 21C).

Glutamate-IR colloidal gold labeling density was estimated in 31 and 36 VACHT-IR motor axon boutons at respectively high and low antibody concentration in tissues sampled from the three animals (Fig. 22). Average glutamate-IR colloidal gold density was significantly higher with the more concentrated antibody (19.5 ± 1.6 SEM compared to 10.2 ± 0.9; p<0.01, Mann-Whitney Rank Sum test) (Fig. 22E, F). Average neuropil
labeling varied in parallel with antibody concentration (9.7 ± 0.7 at high antibody concentration and 6.8 ± 0.7 at low antibody concentration). At high concentration there was some small inter-animal variability in glutamate immunolabeling of VACHT-IR motor axon terminals or neuropil (Fig. 22C, D). However average glutamate-IR was always significantly higher in VACHT-IR motor axon terminals compared to neuropil labeling for each animal individually (p<0.01, Mann-Whitney Rank Sum tests, Fig. 22C) or when pooling data from the three animals together (p<0.001, Mann-Whitney Rank Sum tests, Fig. 22E). At the lower antibody concentration interanimal differences in VACHT-IR motor axon terminals were not significant (Fig. 22D, p=0.147, Kruskal-Wallis One Way Analysis of Variance on Ranks) but basal neuropil labeling was significantly different in between all animals (p<0.001 Kruskal-Wallis One Way Analysis of Variance on Ranks; p<0.05, post-hoc Dunn test for pair-wise comparisons). As a result differences in glutamate labeling between VACHT-IR motor axons and neuropil were statistically significant for animals 1 and 3 (p<0.001, Mann-Whitney Rank Sum test), but not for animal 2 (p=0.669, t-test). Pooled data from all three animals indicated that glutamate-IR inside VACHT-IR terminals was significantly higher than neuropil labeling even at these low labeling conditions (p<0.001, Mann-Whitney Rank Sum test).

At high and low antibody concentrations, average bouton labeling was respectively 2.0 ± 0.2 and 1.7 ± 0.5 times the neuropil staining, the enrichment values calculated at different antibody concentrations were not statistically significant (t-test, p=0.214). Labeling was therefore consistently higher than neuropil, but average glutamate-IR enrichment from neuropil basal level was not as high as with aspartate immunolabeling. Analysis of scatter plots (Fig. 22A, B) suggests this was due to the presence of a portion of synapses
with low or no labeling in both high and low antibody dilutions. Consequently, only 44% and 21% of VACHT-IR motor axon terminals showed glutamate-IR two standard deviations above average neuropil labeling at high and low antibody concentrations respectively.

C-terminals were more consistently immunolabeled with glutamate antibodies (Fig. 21C) compared to aspartate. C-terminals displayed average immunolabeling density significantly higher than neuropil at both antibody concentrations (18.3 ± 1.9 at high and 18.4 ± 2.9 at low concentration). Differences with neuropil labeling were always significant when making comparisons within animals (p<0.001 ANOVA, post-hoc Tukey tests p<0.05) or when pooling data from the three animals together (p<0.001, t-test). C-terminal glutamate labeling was similar to motor axon terminals at high antibody concentrations and even significantly higher at low antibody concentrations. Average enrichment levels from neuropil were similar at both dilutions (2.1 ± 0.2 at the highest concentration of antibody and 2.2 ± 0.3 at the lowest) and these were higher than for C-terminal aspartate immunolabeling. About half of the C-terminals contained immunolabeling density higher than two standard deviations from average neuropil labeling (54.5% at the highest antibody dilution, 45% at the lowest; n=22 C-terminals analyzed at each dilution).

In conclusion, glutamate seems to be similarly enriched with respect to neuropil in VACHT-IR motor axons and C-terminals. However, glutamate content was more variable compared to aspartate in VACHT-IR motor axon synapses.
Figure 13: Low magnification image of “resin sandwich sections” to test aspartate and glutamate antibody specificities. Layers of amino acid enriched brain homogenates are embedded between brain tissue sections. Brain homogenates contain particles or islands with glutaraldehyde-fixed amino acids (arrows). The tissue sections were all 2 \(\mu\)m thick except for the last one which was 4 \(\mu\)m. This thick section gives the orientation necessary to identify the different layers containing different glutaraldehyde-fixed amino acids. Scale bar equals 3 \(\mu\)m.
Fig 14: High magnification images of amino acid enriched islands immunolabeled with aspartate and glutamate antibodies and 10 nm colloidal gold. A-E) Aspartate antibody used at 0.18 µg/ml was applied to resin sandwiches containing islands enriched with amino acids including aspartate, GABA, glutamate, and glutamine. As a control some islands of brain homogenate were not embedded with any amino acids. These blanks served as a control (E,J). Islands embedded with aspartate (A) contained dense immunogold labeling with the aspartate antibody. Islands embedded with other amino acids (B-D) or blank control islands lacking amino acids (E) did not contain a significant amount of immunogold labeling. F-J) Immunolabeling with glutamate antibody at 0.17 µg/ml. Islands embedded with glutamate (H) contained dense immunogold labeling. Weaker immunolabeling was observed in glutamine islands (I). Islands embedded with aspartate (F), GABA (G) or taurine (not shown) did not contain any significant immunogold labeling, nor did the blank or control islands lacking any amino acids (J). Scale bar in J equals 1µm. All images are at the same magnification.
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Figure 15: Quantitative analysis of aspartate and glutamate antibody labeling in resin sandwiches. A-B) Density of labeling of amino acid enriched islands at high (A) and low (B) aspartate antibody dilutions. Filled black dots indicate density estimates for each sampled island. Ten islands were analyzed for each amino acid and antibody dilution. White circles show averaged data. Error bars indicate ± SEM. Dotted line indicates 0 labeling. Only aspartate-enriched islands were significantly immunolabeled above the density of blank islands. C-D) Similar density plots for glutamate immunolabeling at low and (C) and high (D) antibody dilutions. Note differences in labeling density between both antibody dilutions (different y-axis) and also the significant labeling of glutamine islands in addition to glutamate.
Figure 16: Electron microscopy of immunogold-silver labeled VAChT-IR structures and DAB-labeled calbindin-IR dendritic profiles in the spinal cord ventral horn. VAChT-IR is recognized by the relatively large and irregularly shaped electron dense particles in the motoneuron soma, dendrites and synaptic boutons contacting Renshaw cells, as well as in the cholinergic C-terminals contacting motoneurons (see inset). Immunogold-silver electron dense particles could vary significantly in size and density. This is obvious when comparing the two structures labeled at the top of the image (asterisks) and the one at the lower left corner (double asterisks). VAChT-IR immunogold silver deposits were very dense in more superficial terminals (motoneuron terminal in lower left of image). C-terminals were identified as VAChT-IR boutons presynaptic to motoneuron cell bodies and sometimes by the presence of a subsynaptic cistern in the postsynaptic motoneuron (see arrows in inset). Calbindin-IR was revealed with DAB labeling and is visible in Renshaw cell dendrites and terminals. The DAB labeling appears as electron dense diffuse precipitate that forms fine halos around lipid containing membranes of organelles of the cell. Scale bars equal 300 nm.
Figure 17: Aspartate-IR was enriched in VACHT-IR boutons presynaptic to calbindin-IR Renshaw cell dendrites. A) VACHT-IR motoneuron terminals labeled with relatively large silver deposits surround and make synapses with a calbindin-IR Renshaw cell dendrite labeled with DAB. Aspartate-IR 10 nm colloidal gold particles are found inside the VACHT-IR synaptic boutons (arrows) at higher density than in the neighboring neuropil or in the postsynaptic Renshaw cell dendrite. B) Aspartate-IR was sometimes observed on mitochondria (arrows). C, D) Serial sections of the same VACHT-IR motoneuron synapse contacting a calbindin-IR Renshaw cell dendrite revealed a similar level of aspartate immunogold particles in both serial sections of the same terminal. Scale bar equals 300 nm for A and D (C and D are at the same magnification) and 150 nm in B.
Figure 18. Aspartate-IR did not label inhibitory Renshaw cell terminals. A calbindin-IR Renshaw cell terminal establishing a synapse with a large dendrite in LIX (arrow) contained a low density of aspartate-IR 10 nm colloidal gold particles similar to neighboring neuropil. However, a nearby excitatory synaptic bouton (asterisk) contained a relatively larger amount of 10 nm colloidal gold particles. Scale bar equals 300 nm.
Figure 19: Quantification of aspartate-IR revealed enrichment over VACHT-IR motoneuron terminals contacting calbindin-IR Renshaw cells. A,B) Aspartate-IR gold densities in individual VACHT-IR boutons or neuropil at antibody concentrations of 0.36 µg/ml (A) or 0.18 µg/ml (B). VACHT-IR boutons on Renshaw cells or motoneurons are separated according to animal. Each neuropil estimate is from an individual micrograph (2 to 4 areas analyzed per negative). The numbers of boutons or neuropil estimates are indicated. VACHT-IR boutons on Renshaw cells display immunolabeling densities frequently higher than neuropil. Most VACHT-IR terminals contacting motoneurons contain immunolabeling densities similar to neuropil. C-D) Histograms showing average densities at high (C) or low (D) antibody concentrations for each type of terminal and neuropil labeling in each animal. Error bars indicate ± SEM. At both antibody dilutions average aspartate-IR density in VACHT-IR boutons on Renshaw cells was consistently higher than neuropil averages. Variability among animals was noted in labeling intensities. Neuropil labeling was significantly different in mouse 1 compared to mice 2 and 3 at high antibody dilutions and between mice 1 and 2 at low (asterisks in C and D; ANOVA, p<0.001). Average density inside VACHT-IR terminals contacting Renshaw cells was significantly different between mice 1 and 2 at low antibody dilutions (asterisk in D, p<0.001 ANOVA and post-hoc Tukey Test). E, F) Histograms representing pooled density averages from all boutons sampled in the 3 animals at high (E) and low (F) antibody concentrations. Error bars also indicate ± SEM. At both dilutions the average density of aspartate-IR in all 3 animals was significantly higher in VACHT-IR terminals contacting calbindin-IR Renshaw cells than in the neuropil or over
VACHT-IR terminals contacting motoneurons (asterisks, p<0.001 ANOVA and post-hoc Tukey Tests).
Figure 20: Glutamate-IR is enriched in VACHT-IR motoneuron synapses contacting Renshaw cell dendrites. A) Glutamate-IR 10 nm colloidal gold particles (arrowheads) revealed at a high antibody concentration (0.17 µg/ml) were found enriched inside VACHT-IR motoneuron terminals making synaptic contacts (arrows) with a calbindin-IR Renshaw cell. Glutamate-IR gold particles were consistently observed in mitochondria (double arrowheads). B) Two VACHT-IR terminals making synapses (arrows) with a calbindin-IR Renshaw cell show glutamate-IR gold particles (arrowheads) at a reduced density (right bouton in B) or no immunoreactivity (left bouton in B) when immunolabeled using the same glutamate antibody more diluted (0.034 µg/ml). Scale bar in B equals 300 nm. A and B are at the same magnification.
Figure 21: Glutamate-IR is present in excitatory synapses and in C-terminals but not in Renshaw cell synapticboutons. A) Glutamate-IR colloidal gold particles inside a VAChT-IR motoneuron synaptic bouton (labeled with large irregularly shaped electron dense immunogold silver deposits) contacting a calbindin-IR DAB labeled Renshaw cell dendrite. An adjacent excitatory synapses (asterisk) of unknown origin contained higher densities of glutamate-IR gold particles. B) Calbindin-IR Renshaw cell synaptic terminal (containing DAB labeling) making a synapse (arrow) with an unlabeled dendrite. These inhibitory terminals contained glutamate-IR at densities similar or below neuropil labeling. A nearby VAChT-IR terminal making a synaptic contact with a dendrite of unknown origin contains higher levels of glutamate IR gold particles. C) VAChT-IR C-terminals (silver deposits) contacting a motoneuron soma (asterisk) and containing significant levels of glutamate -IR colloidal gold particles. These synapses have a characteristic subsynaptic cistern in the postsynaptic site (see inset, asterisk). Scale bar equals 300 nm in A,B and C.
Figure 22: Quantification of glutamate-IR inside VACHT-IR motoneuron terminals contacting calbindin-IR Renshaw cells and C-terminals. A, B) Dot blots (as in figure 19) showing colloidal gold immunolabeling densities at high (A) or low (B) glutamate antibody concentrations in individual VACHT-IR motoneuron terminals contacting calbindin-IR Renshaw cells, in C-terminals (VACHT-IR terminals on motoneurons) and in the neuropil of the three experimental animals. C, D) Average density of glutamate-IR in each type of synaptic bouton and in the neuropil for each antibody concentration and animal analyzed. Inter-animal variability in glutamate immunolabeling of VACHT-IR motor axon or C-terminals and in neuropil labeling estimates was relatively low using the high antibody concentration (C) but increased when using the low antibody concentration (D). Asterisks indicate significant differences further explained in the results section. E, F) Pooled data average histograms from the 3 mice using glutamate antibodies at 0.17 µg/ml (E) and 0.034 µg/ml (F). Glutamate-IR in VACHT-IR terminals contacting calbindin-IR Renshaw cells was significantly higher compared to neuropil at both antibody dilutions (asterisk indicate p<0.001, ANOVA and p<0.05 post-hoc pair-wise comparisons using a Tukey test). C-terminals showed immunolabeling density similar to VACHT-IR motor axon terminals at high antibody dilutions but significantly higher glutamate immunolabeling density compared to neuropil with low antibody concentrations. In both cases they contained significantly higher colloidal gold densities than the neuropil.
Discussion

Antibody specificity

We confirmed that the aspartate and glutamate polyclonal antibodies used in this study were specific for their targeted amino acids at the concentrations used. Aspartate antibodies exhibited a high level of specificity and on average did not show significant cross reactivity with any of the other glutaraldehyde-fixed amino acids tested. The glutamate antibody appeared to also recognize glutamine, but denser labeling was obtained on glutamate enriched islands than on glutamine enriched islands. Glutamine labeling might in part explain the strong mitochondrial labeling. Cross-reactivity with glutamine is not a confounding factor for this study because 1) immunoreactive density in glutamine islands was approximately half that of glutamate islands, 2) glutamine is the main precursor of glutamate in synaptic boutons and the levels of both of the amino acids are correlated in glutamate-releasing terminals, 3) glutamine is preferentially accumulated in mitochondria and we excluded mitochondria from our density estimates.

Glutamate and aspartate in motor axon synaptic boutons

When comparing aspartate-IR and glutamate-IR in VACHT-IR motor axon boutons making synapses on calbindin-IR Renshaw cell dendrites, it is clear that the density of both excitatory amino acids is significantly greater in the axoplasm of these terminals than in the surrounding neuropil. Of particular interest is the enrichment in aspartate-IR. The majority of boutons showed significant levels of aspartate immunolabeling above neuropil levels independent of the concentration of antibody used. Accumulation of this aspartate inside vesicles will require the presence of specific
transporters different from VGLUTs that do not transport aspartate (Bellocchio et al., 2000; Herzog et al., 2001; Fremeau et al., 2002, Varoqui et al., 2002). Recently, an aspartate transporter, SLC17A5 has been identified in synapses in the CNS (Miyaji et al., 2008). Further experimentation and details of this transporter type will be discussed in Aim 4.

By contrast, glutamate enrichment in the axoplasm of VACHT-IR terminals was present at densities above neuropil labeling only in a population of synapses. Nevertheless, most VACHT-IR motor axon synapses express some level of glutamate-IR that could be made available for synaptic release. Similar to aspartate it would require a transport mechanism other than the currently known VGLUTs for moving glutamate into the vesicles. Interestingly, SLC17A5 is capable of accumulating both aspartate and glutamate (Miyaji et al., 2008), suggesting that this or another similar transporter could co-transport both excitatory amino acids into synaptic vesicles.

Another membrane transporter, SLC10A4, has been recently found to be specifically associated with cholinergic synapses throughout the CNS, but its exact substrate specificity and function have yet to be defined (Geyer et al., 2008). Initial studies with this transporter are also presented in Aim 4.

Co-release of excitatory amino acids with acetylcholine

The electrophysiological data reviewed in the introduction suggest the release of excitatory amino acids and acetylcholine on Renshaw cells in response to activation of motor axons antidromically from the ventral roots. These results can be explained by three different possible mechanisms: 1) some motor axons or collaterals release
excitatory amino acids and others acetylcholine; 2) single motor axon synaptic boutons co-release excitatory amino acids and acetylcholine but each neurotransmitter is packaged in different synaptic vesicles; 3) excitatory amino acids and acetylcholine are co-stored in single vesicles and co-released from them. The first possibility was proposed by Herzog et al., (2004) and is contrary to Dale’s Principle. Our data discredit this possibility because glutamate and aspartate are enriched in VACHT-IR terminals that release acetylcholine. Moreover in Aims 1 and 2 we did not find evidence for a large population of motor axon synaptic boutons lacking VACHT, and the findings of Herzog and colleagues (2004) of VGLUT2 in a portion of motor axons boutons could not be replicated by us (Aim 1 and Mentis et al., 2005) or others (Liu et al., 2009). We therefore conclude that excitatory amino acids and acetylcholine are released from single motor axons.

Unfortunately, using anatomical techniques it cannot be determined if acetylcholine and excitatory amino acids are stored and released from the same or different vesicles. This is because at present, neurotransmitters or transporters cannot be immunolocalized with single vesicle resolution. To investigate this problem it will be necessary to utilize an alternative approach. The most direct test could be obtained by analysis of electrophysiological recordings of miniature excitatory postsynaptic currents (mEPSCs) in Renshaw cells. mEPSCs are assumed to constitute the postsynaptic responses to neurotransmitter release from single vesicles (Del Castillo & Katz, 1954). The binding of excitatory amino acids or acetylcholine to NMDA and AMPA or nicotinic receptors, results in postsynaptic currents with different kinetics (LaMotte d’Incamps & Ascher, 2008), therefore the presence of mEPSC with mixed kinetics revealing
simultaneous opening of nicotinic, AMPA and NMDA receptors would indicate co-release of acetylcholine and excitatory amino acids from single vesicles. In this case there would have to be either a non-specific transporter able to transport both acetylcholine and excitatory amino acids into the same vesicle or alternatively, excitatory amino acid transporters and VAcHT should be colocalized in the same vesicles. If this was not the case, that is excitatory amino acids and acetylcholine are stored in different vesicles, it would open the possibility that single boutons would sometimes operate as a glutamatergic/aspartergic synapse or a cholinergic synapse depending on the random synaptic exocytosis of one or the other type of vesicle. It is unclear what would be the functional advantage for such an operating mode.

Interestingly, glutamate was found at cholinergic C-terminals contacting motoneurons. It appears that glutamate is the primary excitatory amino acid neurotransmitter released along with acetylcholine at these synapses as the amount of glutamate-IR found in these synapses was greater than that of aspartate-IR. The significance of excitatory amino acids at C-terminals is less straightforward than the presence of excitatory amino acids at VAcHT-IR terminals contacting Renshaw cells. This is because the function and C-terminal synaptic mechanisms have yet to be clarified.

**Functional significance**

Although the mechanism for release of excitatory amino acids along with acetylcholine at motoneuron terminals remains unknown, the possible implications of this co-release are of great interest. More specifically, the ability of aspartate to bind to NMDA receptors at postsynaptic sites on the Renshaw cell leads to interesting functional
considerations. Motoneurons and Renshaw cells are tightly coupled and a single motoneuron action potential traveling down a motor axon can evoke high frequency burst firing in the Renshaw cell (Van Keulen 1981). The burst activity is partly explained by the long duration of the underlying excitatory postsynaptic potential in the Renshaw cell. Renshaw cell EPSPs have typical time courses of 50-100 milliseconds (Eccles et al., 1961; Walmsley & Tracey, 1981), much longer in duration than the end plate potential (EPP) observed in muscle (half width of less than 30 ms: Fatt & Katz, 1951). NMDA receptors are known to contribute to the slow component of EPSPs in Renshaw cells (Mentis et al., 2005; Lamotte d’Incamps & Ascher, 2008). Therefore, it seems likely that the activation of postsynaptic NMDA receptors in conjunction with the nicotinic receptors on Renshaw cells could lengthen the EPSP duration and contribute to the generation of the characteristic bursting activity in Renshaw cells. The high aspartate content of these synapses suggests that this excitatory amino acid might preferentially mediate the binding and opening of postsynaptic NMDA receptors at motor axon-Renshaw cell synapses. Unfortunately we are not aware of any experimental procedure to reliably distinguish aspartate from glutamate synaptic release and binding to NMDA receptors at central synapses in situ. Glutamate release is demonstrated by the presence of an AMPAergic component at the motor axon to Renshaw cell synapse (Mentis et al., 2005; Nishimaru et al., 2005; LaMotte d’Incamps et al., 2008). However AMPA currents at these synapses are relatively small and more variable compared to NMDA and this could be explained by the presence of few postsynaptic AMPA receptors or the release of more aspartate than glutamate at the synapse. The localization of AMPA vs. NMDA receptors at this synapse is currently underway in our lab.
Glutamate co-release is observed with many other types of neurotransmitters, as well as with acetylcholine, in the brain and spinal cord and plays a variety of roles in the function of these different synapses. For example, magnocellular basal forebrain neurons release both acetylcholine and glutamate. This colocalization is thought to lead to different time courses in the postsynaptic response with glutamate producing a fast ionotropic response and the acetylcholine producing a slow, G-protein mediated effect via muscarinic receptors (Allen et al., 2006). Synaptosome fractions from the cerebral cortex also co-release glutamate and aspartate with acetylcholine (Docherty et al., 1987). Aspartate alone, not necessarily in conjunction with glutamate, may also be co-released with other neurotransmitters from terminals in the CNS. For example, synapses in the hippocampus exocytose both GABA and aspartate (Gundersen et al., 2004). However, the data shown in this aim are unique in that they constitute the first example known to us that aspartate and acetylcholine may be co-released at a synapse, as compared to synaptosome fractions.

Along with possibly contributing to the time course of the Renshaw cell EPSP and its characteristic bursting activity, co-release of excitatory amino acids and NMDA mechanism may play important roles during the developmental maturation of motoneuron synapses, similar to what has been reported for other brain and spinal cord synapses. For example, co-release of glutamate with GABA and glycine at inhibitory synapses of auditory circuits mediates their maturation (Gillespie et al., 2005). In this system the down regulation of glutamate release and NMDA receptor activation coincides with the change of a mixed GABAergic and glycinergic synapse to a strictly
glycinergic synapse, suggesting a direct effect of NMDA activation with the maintenance of GABA release and/or postsynaptic GABAA receptors.

Moreover, NMDA receptor activation is the major contributor to developmental maturation of glutamatergic AMPA synapses. Calcium entry during NMDA receptor activation is a major mechanism for maturation of these synapses, affecting synthesis, degradation, and postsynaptic recruitment of AMPA receptors (for reviews see Hall & Ghosh, 2008; Kerchner & Nicoll, 2008). It would be interesting, but remains to be explored, if NMDA synaptic activation has similar effects on nicotinic receptor expression, recruitment and degradation.

Alternatively, co-release of excitatory amino acids with acetylcholine at motoneuron synapses contacting Renshaw cells may serve broader developmental functions. One possibility is the pruning and deselection of neuronal targets. Developmentally, motoneurons may establish an excess of connections with several classes of interneurons (in addition to Renshaw cells) and motoneuron targets that could become deselected during development. Synaptic pruning is a generalized mechanism that occurs throughout development in the CNS and the presence of postsynaptic glutamatergic receptors plays a key role in this process (Johnson et al., 1996). High levels of extracellular excitatory amino acids can be responsible for pruning, programmed cell death, and neurite sprouting (Haberecht and Redburn 1996). Moreover NMDA receptor activation modulates dendritic development in motoneurons (Kalb, 1994) and a similar mechanism could operate on Renshaw cells via activation of NMDA receptors postsynaptic to motor axon synaptic boutons.
In the developing Renshaw cell the balance between excitatory inputs from motoneurons and inhibitory inputs from other interneurons appears to be well matched (Gonzalez-Forero, et al., 2005). The mechanism by which this occurs involves heterosynaptic facilitation of inhibitory synapses by motor axon excitatory activity (Gonzalez-Forero et al., 2005), but the exact signaling mechanism has not been studied. The release of excitatory amino acids from motoneuron synapses and postsynaptic activation of NMDA receptors may contribute to modulate inhibitory synaptic strength at Renshaw cells by recruiting inhibitory receptors at inhibitory synaptic sites as has been shown in other neurons (Marsden et al., 2007).

Overall, the synaptic release of excitatory amino acids from motoneurons may have many possible functional roles on Renshaw cells, but it still remains possible that the motoneurons may contain large amounts of these excitatory amino acids as by-products of metabolic processes. In order to determine if they are inside vesicles the presence of an efficient transport system will need to be confirmed. Initial experiments in this direction are presented in Aim 4.
VII. Aim 4: Characterization of the presence of members of the solute carrier (SLC) protein family in the spinal cord ventral horn

Rationale

The lack of VGLUTs at motoneuron synapses contacting Renshaw cells observed in Aim 1 combined with the enrichment of both aspartate and glutamate at these synapses observed in Aim 3 suggests the likelihood that a different mechanism is present for transporting excitatory amino acids into vesicles for release at the synapse. VGLUTs belong to a large family of transporters, namely the solute carrier proteins (SLCs) that are responsible for moving solutes across membranes. Some members of the SLC family have yet to be functionally identified and even when a function has been established in some cells they might express additional functions in other cells or locations. For example, VGLUT1 was first identified as brain-specific Na/Pi cotransporter (BNPI, Ni et al., 1994) and it was not until five years later that its importance in glutamate transport in synaptic vesicles was appreciated because of its sequence similarity to EAT-4, a gene from C. elegans that, when mutated, disrupts glutamatergic neurotransmission (Lee et al., 1999). The whole SLC family includes 43 families and 298 genes (Hediger et al., 2004), leaving many possibilities for the discovery of new transporters and functions.

VGLUTs belong to the SLC17 family, members of this family include several Na+/phosphate co-transporters (SLC17A1-4), a lysosomal H+/sialic acid co-transporter (SLC17A5), the three VGLUTs (SLC17A6-8) and a vesicular nucleotide transporter (VNUTs) (SLC17A9) (for review, Reimer & Edwards, 2004). VGLUTs are specific transporters for glutamate and do not transport aspartate (Bellocchio et al., 2000; Herzog et al., 2001; Fremeau et al., 2002, Varoqui et al., 2002). SLC17A5 was initially related to
the transport of sialic acid in lysosomal membranes (Mancini et al., 1989), but recently was proposed to also function in moving aspartate and glutamate into synaptic vesicles (Miyaji et al., 2008). This study focused on its role in hippocampal synapses and pinealocytes, both known to release aspartate. It is unknown if this transporter is generally responsible for aspartate transport in all types of neurons. The SLC17A5 gene was first identified by its linkage to lysosomal storage genetic diseases (Verheijen et al., 1999). These are characterized by a large amount of free sialic acid excreted in the urine as well as hypotonia, cerebellar ataxia and mental retardation. In the body the sialin transporter / SLC17A5 is expressed in the lung, liver, pancreas, kidney and epithelial tissues; whereas in the CNS intense expression is seen in the hippocampus, striatum, cerebral cortex, Purkinje cells, globus pallidus, some thalamic and brainstem nuclei, and the spinal cord (Yarovaya et al., 2005; Miyaji et al., 2008). Interestingly, the sialin, or SLC17A5 transporter shows highest amino acid sequence homology with the VGLUTs (SLC17A6-8) (Verheijen et al., 1999; Yarovaya et al., 2005) which led to it being tested as a possible aspartate transporter (Miyaji et al., 2008). The recent designation of SLC17A5 as a novel excitatory amino acid transporter in synaptic vesicles makes it a potential candidate for possible VGLUT-independent loading of glutamate and aspartate in motor axon synaptic boutons that contain VAcT-IR, but lack VGLUT-IR. If found at motoneuron synapses on Renshaw cells, it might provide a potential substrate for the release of neurotransmitters that can act on postsynaptic AMPA/NMDA receptors expressed by Renshaw cells.

Although not closely related to VGLUTs or SLC17A5, a second potential candidate is SLC10A4. This transporter was recently cloned (Splinter et al., 2006) and is
shown to be expressed in cholinergic neurons and is transported to their synaptic varicosities (Geyer et al., 2008). This transporter belongs to the SLC10 family of sodium bile acid symporter related proteins, however no transport of any substrate related to this family has been found related to SLC10A4 (Splinter et al., 2006; Geyer et al., 2008). At present it is an “orphan” transporter of unknown function. While other members of the SLC10 family are distributed in tissues throughout the body with little expression in brain, SLC10A4 expression is highest in neural tissue (Splinter et al., 2006; Geyer et al., 2008). Specifically, in the brain SLC10A4 is found in dopaminergic neurons of the substantia nigra and ventral tegmentum and their terminals (Jørgensen et al., 2006; Zhou et al., 2009). SLC10A4 is also expressed in cholinergic neurons and their terminals in the basal forebrain and in the spinal cord (Geyer et al., 2008). It is known that at least glutamate is co-released from ventral tegmental dopaminergic neurons (for review, Lapish et al., 2006) and from basal forebrain cholinergic neurons (Allen et al., 2006). Although the function of SLC10A4 is yet unknown, its presence in the synaptic boutons of cholinergic and dopaminergic neurons that co-release excitatory amino acids makes it a potential candidate for transport of excitatory amino acids at motoneuron synapses contacting Renshaw cells.

Therefore, as a first tentative approach to investigate some potential transporter candidates, we studied in this aim the localization of SLC10A4 and SLC17A5 in the synaptic boutons established between motor axons and Renshaw cells. For this purpose we performed triple immunolabeling experiments to localize these transporters inside VAcT-IR synapses on calbindin-IR Renshaw cells. We first used antibodies previously characterized and kindly donated by Dr. Yoshironi Moriyama (SLC17A5; Okayama
University School of Medicine, Dentistry, and Pharmaceutical Sciences, Japan) and Dr. Joachim Geyer (SLC10A4, Justus Liebig University of Giessen, Germany). We could not replicate any positive immunostaining with Dr. Moriyama’s SLC17A5 antibody and therefore we used a different commercially-available antibody against “sialin”. We only tested these antibodies against SLC17A5 in the spinal cord where the distribution of sialin / SLC17A5 is at present unknown. Therefore these immunolocalization studies are the first analyses of the spinal cord distribution of this transporter. The distribution and patterns of immunoreactivity for SLC10A4 using the well-characterized antibody provide to us by Dr. Geyer perfectly replicated the distributions reported for the spinal cord in the original paper (Geyer et al., 2008).
**Materials and methods**

One wild type young adult mouse (6 weeks old) was transcardially perfused as described in Aim 1 with a 4% paraformaldehyde solution in 0.1M PB. The spinal cord was removed and placed in a 4% paraformaldehyde solution for 2 hours, and then transferred to a 15% sucrose solution overnight. Transverse sections of lumbar 4 and 5 segments of the spinal cord were then cut at 50µm using a freezing sliding microtome, and collected in 0.01M PBS. Triple immunofluorescence experiments were performed using antibodies for either SLC10A4 (rabbit, 1:1000, gift from Dr. J. Geyer, Justic Liebeg University of Giessen, Germany) or SLC17A5 (anti-rabbit, 1:2000, Abcam) with VAChT (goat, 1:1000) and calbindin D28K (rabbit, 1:1000) antibodies in a similar manner as described in Aim 1.

**Confocal analysis of SLC10A4 and SLC17A5 contacts on Renshaw cells**

Images were collected using an Olympus Fluoview FX1000 system confocal microscope for tri-color images using excitations at 488nm, 568nm, and 633nm. A sequential imaging mode was used to acquire each fluorescent line independently and to minimize cross-talk. Series of high magnification (60x2.5, NA 1.4) confocal optical images (0.5 µm z-steps) throughout calbindin-IR Renshaw cells in ventral LVII and of motoneurons in LIX, receiving VACHT contacts were imaged. The presence of SLC10A4 or SLC17A5 was examined using Fluoview software.
Results

**SLC10A4-IR in the spinal cord**

Spinal cord sections were triple immunolabeled for SLC10A4, VAChT, and calbindin. As observed in Aim 1, VAChT-IR punctae were found in the superficial lamina of the dorsal horn, in large motoneuron somas in LIX, in the C-terminals contacting motoneurons, and in the motoneuron contacts on calbindin-IR Renshaw cells in the ventral portion of LVII. SLC10A4-IR, like VAChT-IR reveals intensely labeled punctae in these same areas in the dorsal and ventral horn (Fig. 23 A-C). Specifically in LIX, VAChT-IR and SLC10A4-IR were colocalized in the C-terminals contacting motoneurons. Similar to VAChT-IR in motoneurons, SLC10A4-IR is also weakly expressed in the somas, with small punctae observed throughout the cytoplasm, but with more intense labeling observed in the terminals contacting Renshaw cells. The VAChT-IR punctae contacting Renshaw cell somas and dendrites were also colocalized with SLC10A4-IR. However, the Renshaw cells also receive contacts from punctae that are SLC10A4-IR and lack VAChT-IR (Fig. 23). Therefore, some of the SLC10A4-IR contacts on Renshaw cells may not originate from motoneurons. However, a qualitative analysis of the VAChT-IR terminals contacting Renshaw cells shows that almost all are SLC10A4-IR.

**SLC17A5 in the spinal cord**

Triple immunolabeling experiments were also used for SLC17A5 along with VAChT-IR and calbindin-IR to label motoneuron and Renshaw cells, respectively. In low magnification confocal images, SLC17A5-IR intensely labels motoneuron somas in
LIX in the ventral horn. In LIX, the appearance of SLC17A5-IR was quite similar to the appearance of ChAT-IR in the motoneurons observed in Aim 2, with brightly labeled punctae filling the cytoplasm of the motoneuron somas. Occasionally, some structures with glial-like appearance were observed to have SLC17A5-IR in the spinal cord, but there were no other specific pools of neurons labeled outside of LIX (Fig. 24).

SLC17A5-IR punctae occurred at very low density in the Renshaw cell area of LVII. However, unlike the SLC10A4-IR colocalizing with VACHT-IR in terminals contacting Renshaw cells, there was no apparent colocalization of SLC17A5-IR with VACHT-IR at the motoneuron synapses contacting Renshaw cells (Fig. 24). The VACHT-IR C-terminals contacting motoneurons also appeared to lack a significant amount of SLC17A5-IR (Fig. 24).
Figure 23: Co-localization of SLC10A4 with VACHT-IR synapses in motor axons and C-terminals. A) Low magnification confocal image of a lumbar spinal cord section immunostained for SLC10A4 (Cy3, red), VACHT (FITC, green) and calbindin (Cy5, blue). A1 shows the distribution of SLC10A4. SLC10A4-IR appeared as punctae with higher densities in superficial laminae I and II of the dorsal horn (arrows), in lamina IX (LIX, region enclosed by white dashed line) and the Renshaw cell area (area enclosed by yellow line). Motoneuron cell bodies and some interneurons close to the central canal (arrowhead) contained some weak SLC10A4-IR. A2 shows the same field with VACHT-IR boutons (green) and calbindin-IR neurons superimposed (blue). Many VACHT-IR terminals target LIX (C-terminals) or calbindin-IR cells in the Renshaw cell area (motor axon synapses). A3 shows these two immunoreactivities superimposed with SLC10A4. There is abundant co-localization between VACHT-IR boutons and SLC10A4 (yellow) in LIX and the Renshaw cell region. B) Higher magnification images of SLC10A4-IR in the ventral horn (B1), VACHT and calbindin-IR together in the same field (B2) or all three immunoreactivities superimposed (B3). Many punctae in close apposition to calbindin-IR Renshaw cell dendrites (blue) colocalize VACHT-IR and SLC10A4 (yellow). C) High magnification image of calbindin-IR Renshaw cell and dendrites (blue) receiving contacts from SLC10A4 punctae (arrowheads in C1) and VACHT-IR boutons (arrowheads in C2). Superimposition of all three immunoreactivities demonstrates that many VACHT-IR contacts co-localize with SLC10A4 (yellow arrowheads in C3). A few SLC10A4 boutons in contacts with Renshaw cell dendrites did not contain VACHT-IR (red arrowheads in C3). D) Optical cross-section through a motoneuron cells body weakly labeled for SLC10A4 (D1) and VACHT (D2). The cell body is surrounded by
boutons that are SLC10A4 (arrowheads in D1) and VACHT (arrowheads in D2) immunoreactive. D3 show superimposition of both immunoreactivities. Co-localization is indicated by yellow arrowheads. Scale bars, A, 250 μm; B, 100 μm; C and D, 20 μm.
Figure 24: Distribution of SLC17A5 in the spinal cord. A) Low magnification image of SLC17A5 immunoreactivity (Cy3, red) superimposed with calbindin-IR (Cy5, blue) in A1 and in addition with VACHT-IR in A3. A2 shows distribution of VACHT-IR boutons nad calbindin-IR cells in the same field. Motoneurons in lamina IX (LIX, white dashed line) express abundant cytoplasmatic immunoreactivity for SLC17A5. There are few punctae immunolabeling resembling putative synaptic boutons. Very few are localized inside the Renshaw cell area (yellow line). In addition, many cells with morphological features similar to glial cells contain SLC17A5 immunoreactivity (arrows in A1). B) High magnification images of the ventral horn showing immunoreactivite patterns inside motoneurons (red cells) and in the regions occupied by calbindin-IR Renshaw cells (blue). Many motoneuron are heavily immunostained in the cytoplasm with the SCL17A5 antibody. Superimposition with VACHT-IR (B3) demonstrates lack of co-localization either around motoneuron cell bodies or in contacts on Renshaw cells. C) High magnification of calbindin-IR Renshaw cells and dendrites (blue) with SLC17A5 immunoreactivity (C1). Very few SLC17A5-IR punctae appear to contact Renshaw cell dendrites (red arrow in C1). In contrast, many VACHT-IR boutons contact Renshaw cells dendrites (green arrows in C2). SLC17A5-IR and VACHT-IR contacts are not co-localized (C3). Scale bars, A, 250 µm; B, 100 µm; C, 20 µm.
Discussion

The lack of known VGLUTs at motoneuron synapses contacting Renshaw cells along with the presence of related transporters in motoneurons and their synapses suggests that there may be alternative mechanisms for transporting aspartate and glutamate into synaptic vesicles. The immunocytochemistry data presented in this aim indicate that SLC10A4, an as of yet functionally uncharacterized member of the solute carrier protein family, is present in VAcChT-IR synaptic vesicle clusters presynaptic to Renshaw cells. However, the putative aspartate/glutamate transporter SLC17A5 is not. Although SLC17A5 is highly expressed by motoneurons it is not present in their presynaptic terminals.

SLC10A4: a putative aspartate/glutamate transporter?

The localization evidence presented here agrees with the data published by Geyer et al. (2008) and favors SLC10A4 as having a role in synaptic neurotransmission at motor axon synapses on Renshaw cells. SLC10A4 exhibited an immunolabeling pattern in the spinal cord that was quite similar to that of VAcChT immunolabeling, with much colocalization observed. Although a significant number of SLC10A4-IR punctae lacked VAcChT-IR, almost all VAcChT-IR boutons on Renshaw cells and around motoneurons (C-terminals) contained SLC10A4. This co-localization gives evidence for SLC10A4 presence in synaptic vesicles; however future electron microscopy and electrophysiological experiments would be necessary to confirm its location in synaptic vesicles and its function at the motoneuron synapses on Renshaw cells.
By itself SLC10A4-IR colocalization with VACHT-IR in boutons presynaptic to Renshaw cells does not alone prove that this is the molecule responsible for transporting aspartate and glutamate into vesicles. However, evidence from other brain regions gives further support to this possibility. SLC10A4 is also present in other cholinergic synapses in the CNS known to co-release glutamate and acetylcholine. The diagonal band of Broca and the medial septum in the basal forebrain send projections to the hippocampus that co-release acetylcholine and glutamate (Huh et al., 2008). Similar cells in the basal nucleus of Meynert project to the neocortex and cholinergic synaptosome fractions from neocortex also release glutamate and aspartate (Docherty et al., 1987). All these cholinergic neurons of the basal forebrain express SLC10A4 in their synaptic boutons (Geyer et al., 2008). Damage to these neurons decreases cognitive function and memory and has been associated with Alzheimer’s disease (reviewed in Muir et al., 1997).

Interestingly, close examination of in situ hybridization and immunocytochemistry data in the literature reveals that neurons in this region do not contain any of the known VGLUTs (see Herzog et al., 2001; Varoqui et al., 2002; Gras et al., 2002; Boulland et al., 2004). Herzog and colleagues (2001) did show VGLUT1 expression in the lateral septum but none in the medial septum. Similar to the controversy found in the spinal cord literature one laboratory reported the presence of VGLUT3 in the somata of neurons in the basal forebrain, but they were unable to detect them at the synapses (see Gritti et al., 2006). The presence of VGLUT3-IR in somata but not in the axon terminals is difficult to interpret. It might suggest possible non-synaptic release from dendrites and somata as originally described by Fremeau et al. (2002) in other neurons and in glia, which also express this transporter. In any case, it does not explain the mechanism of
glutamate/acetylcholine co-release from the synaptic terminals of cholinergic forebrain neurons. Similar to our results in Aim 1 and the different results obtained by other authors on VGLUT expression in nociceptors, VGLUTs might be undetectable in forebrain cholinergic neurons because of low expression. The use of amplification techniques and contrasting in situ hybridization data from various labs using more sensitive riboprobes will definitely be needed to clarify this issue. Alternatively it is possible that transport of glutamate or aspartate in basal forebrain cholinergic neurons is mediated by another transporter shared with motoneurons, such as SLC10A4. In this context it is interesting that SCL10A4 was also detected in the terminal fields of nociceptor synapses in dorsal horn laminae I and II. Further studies will be necessary to corroborate if these are the sensory afferents in which VGLUT2 has been shown to be detectable at very low levels (see discussion in aim 3).

SLC10A4 is also highly expressed in mesencephalic dopaminergic neurons of the substantia nigra and ventral tegmentum (Jørgensen et al., 2006; Zhou et al., 2009; Geyer et al., 2008). Progressive degeneration of these neurons is associated with Parkinson’s disease. Neurons in these areas also co-release glutamate (Lapish et al., 2006). Some of the neurons in the substantia nigra contain VGLUT2 (Varoqui et al., 2002), however, the substantia nigra contains a heterogeneous pool of neurons and VGLUT2 mRNA has been detected in as few as 0.1% or up to 18% of dopaminergic neurons (tyrosine-hydroxylase positive) (Kawano et al., 2006; Yamaguchi et al., 2007; Dal Bo et al., 2008). VGLUT2 expression is higher in mesencephalic dopaminergic neurons during embryonic development, primary cultures or after neurochemical lesion. Nevertheless, in the adult and in vivo, most do not express VGLUTs despite functional evidence of glutamatergic
actions from their synaptic terminals (Chuhma et al., 2004; Lavin et al., 2005).
Interestingly the largest density of SCL10A4-IR reported in the brain occurs in the projection area of dopaminergic substantia nigra cells in the caudate-putamen (Geyer et al., 2008). It is therefore possible that similar to cholinergic neurons of the basal forebrain and motoneurons these neurons co-release glutamate using a VGLUT-independent mechanism. The presence of SLC10A4 in all these neurons is highly suggestive of a role in excitatory amino acid release.

Unfortunately SLC10A4 is a member of the family of bile acid transporters of unknown function. Failure to demonstrate transport of bile acids classifies SLC10A4 as an orphan transporter. SLC10A4 was only recently cloned and found primarily in the brain (Splinter et al., 2004; Geyer et al., 2008). It is composed of 437 amino acids, 7 transmembrane domains, an extracellular N-terminal and an intracellular C-terminal. It lacks however a motif common to other bile acid transporters in the family (SCLA10A1-3). Structurally, SLC10A4 is still classified as an organic anion transporter (Splinter et al., 2004) and therefore theoretically could carry the negatively charged aspartate and glutamate at physiological pH.

**SLC17A5**

Whereas SLC10A4 was found at motoneuron terminals contacting Renshaw cells, the expression of SLC17A5-IR was restricted to motoneuron somata and was not detected in their synapses. There are two possible reasons for this: 1) SLC17A5 expression is restricted to the soma and not present in the terminals, or 2) SLC17A5 is expressed at too low of a level to be detected using standard immunocytochemistry.
techniques. SLC17A5 is known to transport aspartate and glutamate into synaptic vesicles in hippocampal cells as well as in pinealocytes (Miyaji et al., 2008). However, when it was originally cloned it was characterized as a lysosomal transporter for sialic acid and glucuronic acid (Mancini et al., 1989; Verheijen et al., 1999). The lack of SLC17A5 in motoneuron terminals, but its presence in the motoneuron soma suggests that in these neurons SLC17A5 might have only a functional role in lysosomes.

Lysosomal sialic acid transport of SLC17A5 have been related to the processing and turn-over of glycoproteins, many of which are present on the membrane of neurons. The motoneurons are exceptionally large compared to other neurons and the large amount of membrane surrounding the soma may indicate a very active turnover via endocytosis of membrane proteins containing sialic or glucuronic acid. The lack of expression of SLC17A5 in motoneuron terminals, but its expression in the terminals of hippocampal neurons and pinealocytes suggests that SLC17A5 may have two functions in the central nervous system 1) for lysosomal transport and 2) for vesicular transport of excitatory amino acids. Different functions might be expressed differently in different neurons. It also suggests that there should be specific mechanisms for the targeting of SLC17A5 to lysosomal or synaptic vesicle membranes. Finally the possible presence of a variety of transporters from different SLC families capable of aspartate and/or glutamate transport into synaptic vesicles suggests a diversity of functions and possible regulatory mechanisms for glutamate and aspartate release in addition to the known functional modulation of VGLUTs.
VIII. General conclusions and discussion

In this thesis, I hypothesized that excitatory amino acids are released with acetylcholine at central motoneuron synapses contacting Renshaw cells. In regard to this hypothesis there were several novel findings within this thesis.

1. Vesicular glutamate transporters (VGLUTs) are not present in motoneuron terminals contacting Renshaw cells.

2. Motoneurons are the only likely source of cholinergic input on Renshaw cells and the Hb9:EGFP mouse model is a valuable transgenic model for examining motoneuron contacts on Renshaw cells at early postnatal ages.

3. Glutamate-IR and aspartate-IR is enriched in the axoplasm of motoneuron synapses on Renshaw cells.

4. One member of the solute carrier protein family, SLC10A4, appears to colocalize with synaptic vesicle pools of motoneurons (labeled with VACHT). No evidence for the putative aspartate transporter (SLC17A5) was found at these synapses.

The data presented suggest that motoneurons likely co-release acetylcholine, aspartate and glutamate. Both amino acid neurotransmitters are accumulated in synaptic terminals but lack a known vesicular transporter system. We identified a putative transporter, SLC10A4 that colocalizes with VACHT-IR punctae. Future work is necessary to characterize the substrate of the transporter and loss of function experiments (knockout, siRNA experiments, etc.) will need to be performed to demonstrate its possible role in excitatory amino acid release.
If confirmed this carrier system would constitute an important vesicular transporter responsible for VGLUT-independent release of aspartate and glutamate. This information would be key for a better understanding of the function of motoneuron synapses. In addition it might have broad significance since similar mechanisms could then operate in basal forebrain cholinergic neurons and substantia nigra dopaminergic neurons. Both of these sets of neurons lack VGLUTs but are known to release excitatory amino acids and are involved in neuropathologies like Alzheimer’s disease (cholinergic) and Parkinson’s disease (dopaminergic).
IX. Significance

The co-release of excitatory amino acids and acetylcholine from motoneuron terminals may have far reaching physiological implications. This co-release may contribute to the characteristic long duration bursting activity of Renshaw cells. Motoneuron synapses on muscle targets in the periphery have EPPs that are much shorter in duration than the EPSPs recorded from Renshaw cells (Fatt & Katz, 1951; Eccles et al., 1961; Walmsley & Tracey, 1981). The slow component of the EPSPs in Renshaw cells is attributed to the activation of NMDA receptors (Mentis et al., 2005; Lamotte d’Incamps & Ascher, 2008). The co-activation of both NMDA receptors and cholinergic receptors following neurotransmitter release from motoneurons may underlie the long duration bursting activity of Renshaw cells that isn’t observed in muscles in the periphery.

Historically, motoneuron synapses have been thought of as having strictly cholinergic functions. Therefore, it is possible that motoneurons have targets other than Renshaw cells that may lack cholinergic receptors, but are still able to receive input from motoneurons via glutamatergic receptor activation. For example, in work with our collaborators (Mentis et al., 2005) cholinergic blockers alone did not greatly inhibit locomotor-like activity following sciatic nerve stimulation, whereas the addition of glutamatergic receptor antagonists completely blocked this activity. This suggests that interneurons other than Renshaw cells, may receive input from motoneurons, but lack cholinergic receptors. Having more than one neurotransmitter released from motoneurons allows for the activation of more than one type of postsynaptic receptor and thus allows for the activation of interneurons that have different postsynaptic receptors.
present in their membranes: either cholinergic and glutamatergic/aspartergic receptors or only cholinergic or only glutamatergic receptors.

The co-release of excitatory amino acids and acetylcholine may also contribute to the maturation of developing motoneuron synapses. Postsynaptically, NMDA receptor activity is known to recruit AMPA receptors to the synapse (Hall & Ghosh, 2008; Kerchner & Nicoll, 2008). To date, it is not known whether the NMDA receptors are able to also recruit cholinergic receptors to the synapse. Future studies examining the mechanism for recruitment of cholinergic receptors to motoneuron synapses on Renshaw cells is needed to better understand the role that NMDA receptors at these developing synapses.
X. References


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