Making Memories Stronger: Characterising the Effect of Piracetam, Memantine and Brain-Derived Neurotrophic Factor on Consolidation and Reconsolidation in the Day-Old Chick

Submitted by

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Thesis Abstract

This series of studies investigates the memory-enhancing properties of two drugs, piracetam (an AMPAkine drug) and memantine (an uncompetitive NMDA receptor antagonist), as well as brain-derived neurotrophic factor (BDNF; a neurotrophin), using the passive avoidance learning (PAL) task in the day-old chick. Piracetam and memantine have both been demonstrated to enhance cognition under a variety of circumstances, and recent research has indicated a role for BDNF in long-term potentiation as well as memory function.

It was found that a 10 mg/kg dose of piracetam facilitated memory recall at 3 hours post-training when injected from immediately after until 120 minutes after weak training in the PAL task. Administration of 10 mg/kg piracetam, 0.1 mM memantine or 12.5 μg/kg recombinant BDNF immediately after training led to memory facilitation which lasted for up to 24 hours following training. These three substances also facilitated recall at 3 hours post-reminder when administered in conjunction with a reminder trial, the purpose of which was to induce recall. These findings indicate the enhancement of both consolidation and reconsolidation of healthy memory in a weak training task by piracetam, memantine and BDNF.

It was concluded that, with respect to the Gibbs and Ng three-phase model of memory, piracetam appears to facilitate at least the long-term memory phase (although additional effects at earlier phases cannot be ruled out), while memantine and BDNF may act at the short-term or possibly later phases of memory.

Statement of Authorship

Except where reference is made in the text of the thesis, this thesis contains no material published elsewhere or extracted in whole or in part from a thesis submitted for the award of any other degree or diploma.

No other person's work has been used without due acknowledgement in the main text of the thesis.

The thesis has not been submitted for the award of any degree or diploma in any other tertiary institution.

Chapters 2 to 4 of this thesis consist of works of joint authorship. In all instances, I am the primary author and have made the most substantial contribution to the work presented, including data collection, data analysis and preparation of scientific articles. The contributions of the other authors are as follows: Professor Simon Crowe – research supervision, project conception and experimental design; Ms Agnes Hazi – drug preparation; Ms Leslie Schachte – assistance with data collection.

All research procedures reported in this thesis were approved by the La Trobe University Animal Ethics Committee (approval numbers AEC10-48 and AEC07-39-P).

Signed: _____ Date: _____

Alternate Thesis Format

This thesis has been written in the alternate format – that is, Chapter 1 is an overall introduction to the thesis, Chapters 2 to 4 are empirical research papers written as stand-alone journal articles, and Chapter 5 is a discussion chapter that brings together the content of the papers in the preceding three chapters, set in the context of Chapter 1. As per the requirements of submission in the alternate format, a minimum of one (1) paper has been accepted for publication in a peer-reviewed scientific journal (see Chapters 3 and 4). Chapters 2 to 4 are presented exactly as they have been submitted to their respective journals, with two exceptions – figure numbering has been altered to include the relevant chapter number, and the referencing format has been changed in order to adhere to APA style guidelines. The author list and journal submission details are included on the first page of each of Chapters 2 to 4.

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Publications and Presentations Arising from this Thesis

Publications

Samartgis, J. R., Schachte, L., Hazi, A. & Crowe, S. F. (2012). Memantine facilitates memory consolidation and reconsolidation in the day-old chick. *Neurobiology of Learning and Memory, 97*(4), 380-385. doi: 10.1016/j.nlm.2012.02.009

Samartgis, J. R., Schachte, L., Hazi, A. & Crowe, S. F. (2012). Brain-derived neurotrophic factor facilitates memory consolidation and reconsolidation of a weak training stimulus in the day-old chick. *Neuroscience Letters, 516*(1), 119-123. doi: 10.1016/j.neulet.2012.03.071

Oral Presentation

Postgraduate Research Symposium of the Australian Psychological Society's College of Clinical Neuropsychologists [Victorian Section], Melbourne, 2 September 2011. Samartgis, J. R., Schachte, L., Hazi, A., & Crowe, S. F. Piracetam enhances memory consolidation and reconsolidation in the day-old chick.

Poster Presentation

Inaugural Student Brain Symposium of the Students of Brain Research, Melbourne, 6 October 2011. Samartgis, J. R., Schachte, L., Hazi, A., & Crowe, S. F. Memantine enhances memory consolidation and reconsolidation in the day-old chick.

Chapter 1: Introduction

This thesis will focus on two nootropic drugs each of which has a different mechanism of action: piracetam, an "AMPAkine" drug (so named due to its facilitatory action at α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid [AMPA] receptors), and memantine, a non-competitive *N*-methyl-D-aspartate (NMDA) receptor antagonist. AMPA and NMDA glutamate receptors are both intimately involved in hippocampal long-term potentiation (LTP), which is believed to be a key neurophysiological process in the formation and consolidation of memory (e.g. Morris, Anderson, Lynch, & Baudry, 1986; Whitlock, Heynen, Shuler, & Bear, 2006). Therefore, these two drugs have been chosen in order to examine the effect of modifying the function of either of two types of glutamate receptors on memory formation. Moreover, both piracetam and memantine have been reported to be well tolerated at low doses, with few behavioural side effects in experimental animals (e.g. Mondadori & Petschke, 1987; Wise & Lichtman, 2007). The role of brain-derived neurotrophic factor (BDNF; a member of the neurotrophin family of endogenous proteins) in memory processing will also be explored here, given the emerging evidence suggesting its facilitatory role in LTP (McAllister, Katz, & Lo, 1999; Poo, 2001) and in memory formation (Tyler, Alonso, Bramham, & Pozzo-Miller, 2002; Yamada, Mizuno, & Nabeshima, 2002). Another crucial issue in contemporary memory research – namely, reconsolidation – will also be examined, followed by a discussion of the methodology employed in this series of experiments (i.e. the use of the passive avoidance learning [PAL] task in the day-old domestic chick).

The Mechanics of Synaptic Transmission

Given that LTP is widely considered to be involved in memory formation (e.g. Morris et al., 1986; Whitlock et al., 2006), it will form the basis of a substantial proportion of this discussion. However, before a discussion of LTP can take place, an understanding of the non-plastic aspects of synaptic transmission is required. The

following is a brief overview of the functions of relevant neurotransmitters and receptors in this process (for further detail, see Bear, Connors, & Paradiso, 2006; Carlson, 2009):

Synaptic transmission is the means by which neurons communicate with one another. Neurotransmitter molecules (e.g. glutamate) are released from the pre-synaptic neuron where they cross the synaptic cleft, then bind to and, in some cases, activate receptors on the post-synaptic neuronal membrane. A neurotransmitter may exert a direct or indirect effect upon the receptor to which it binds. Activation of receptors causes a cascade of events to occur, which results in either excitation or inhibition of the post-synaptic neuron, depending on the neurotransmitter(s) released and the types of receptors on the post-synaptic membrane. The subtype of LTP that will form the focus of this discussion primarily involves the neurotransmitter glutamate and AMPA and NMDA receptors and is concerned with post-synaptic neuronal excitation.

AMPA receptors are ionotropic (i.e. their action involves the passage of ions through a pore in the receptor itself), ligand-gated (i.e. activation is achieved by the binding of a neurotransmitter – in this case, glutamate), and are found extensively throughout the central nervous system. Each receptor is made up of four subunits, and each subunit includes a separate receptor binding site. Upon activation by glutamate (i.e. its attachment to the active binding site), the receptor complex undergoes a conformational change, causing its ion pore to open. This allows the passage of ions from the synaptic cleft into the post-synaptic neuron. Primarily Na^+ ions pass through the pore, although in some cases other ions may be admitted depending on the specific subunit composition of the receptor. During synaptic transmission, AMPA receptors on the post-synaptic membrane are responsible for a degree of cell depolarisation, which results from the cation (positive ion) influx that occurs when the receptors are activated – this is called the excitatory post-synaptic potential (EPSP). Multiple EPSPs may be summed in

order to result in large-scale cell depolarisation and thus stimulation of the post-synaptic neuron.

Like AMPA receptors, NMDA receptors are also ionotropic, ligand (glutamate)-gated, and found on post-synaptic neuronal membranes. Unlike AMPA receptors, however, they are also voltage-gated (i.e. activation of NMDA receptors occurs following a change in membrane electrical potential; this is *in addition to* the binding of glutamate). When bound by glutamate, the ion pore of an NMDA receptor is blocked by an Mg^{2+} ion. Once the cell depolarisation resulting from AMPA receptor activation (i.e. many summed EPSPs) reaches a certain critical level, the Mg^{2+} ion is expelled into the synaptic cleft and Na^{+} and Ca^{2+} ions are able pass through the pore of the NMDA receptor. The Ca^{2+} ions that enter the post-synaptic neuron have functions relating to the mechanism of LTP, which will be elaborated upon in the following section.

Note that an additional class of glutamate receptors also plays a role in synaptic transmission (and indeed, LTP and memory) – namely, G protein-coupled metabotropic glutamate receptors (MGluRs). These receptors, rather than being an ion channel and allowing the passage of ions in and out of the cell (cf. ionotropic receptors), produce their effect by signalling an intracellular biochemical cascade of events upon ligand binding. Recent research has shown that blockade of group I MGluRs impairs memory consolidation and reconsolidation in the day-old chick (Gieros, Sobczuk, & Salinska, 2012; Salinska, 2006). Thus, MGluRs appear to be necessary for both memory consolidation and reconsolidation; however, it should be noted that this thesis will focus on the action of ionotropic glutamate receptors since these are the sites of action of the drugs under consideration here.

Long-Term Potentiation and Memory

In a seminal study conducted almost four decades ago, Bliss and Lømo (1973) demonstrated the potentiation of synapses in rabbit hippocampi following electrical

stimulation. They noted that after repetitive high-frequency stimulation of neurons in the perforant pathway (pre-synaptic cells), granule cells in the dentate gyrus (post-synaptic cells) displayed a much larger EPSP to subsequent single-pulse stimulation than was observed prior to the high-frequency stimuli trains, and that this potentiation of EPSPs could last for a period of up to 10 hours. Shortly after this, Douglas and Goddard (1975) christened this phenomenon “long-term potentiation”.

Since then, a more defined and elaborated model of NMDA-receptor-dependent hippocampal LTP has been developed (for details, see Baudry, Bi, Gall, & Lynch, 2011; G. Lynch & Baudry, 1984; M. A. Lynch, 2004; Sweatt, 1999). Hippocampal LTP is the focus here due to the widely accepted view that the hippocampus is crucially involved in the formation of many types of memory (e.g. Squire & Zola-Morgan, 1991). As the neurochemical processes that take place in the context of LTP are many and complex, only some of the key processes will be introduced here.

LTP is described as having three distinct phases: initial LTP, early LTP and late LTP (Sweatt, 1999). Very little is known about initial LTP (also called short-term potentiation), so this discussion will be restricted to early and late LTP. The model described here will focus primarily on post-synaptic events, given that the majority of findings point toward post-synaptic receptor function as the critical ingredient for LTP induction (Sweatt, 1999), although it should be noted that pre-synaptic changes have also been described in LTP, such as increased neurotransmitter release (M. A. Lynch, 2004).

As has been described thus far, the influx of Na^+ ions via AMPA receptors leads to cell depolarisation — the EPSP. Whilst a single EPSP is insufficient in itself to result in the induction of early LTP, many summed EPSPs (e.g. resulting from a high-frequency train of stimuli) achieve this by causing enough cell depolarisation to activate NMDA receptors and to allow the influx of Ca^{2+} ions. The function of these ions is to cause the activation of various calcium-dependent protein kinases — in particular,

calcium/calmodulin-dependent protein kinase II (CaMKII) and protein kinases A and C (PKA and PKC). These kinases become autonomously active and then cause the phosphorylation (and therefore, activation) of mitogen-activated protein kinases (MAPKs), of which a subset are called extracellular signal-regulated kinases (ERKs). Early LTP is maintained via the persistent activation of calcium-dependent protein kinases, which are responsible for phosphorylation of existing AMPA receptors, leading to heightened receptor activity, and insertion of new AMPA receptors into the post-synaptic membrane. These two events constitute early LTP expression (e.g. H. K. Lee, Barbarosie, Kameyama, Bear, & Huganir, 2000; Malinow & Malenka, 2002).

An important difference between early and late LTP is that the latter process involves gene transcription and protein synthesis, whereas the former does not. Late LTP is induced by the persistent activation of ERK. Late LTP is maintained by the phosphorylation of nuclear transcription factors (for example, cyclic-AMP response element-binding protein [CREB]) by ERK and other kinases, leading to the increased synthesis of molecules such as protein kinase Mζ (PKMζ), a calcium-independent protein kinase (Kelly, Crary, & Sacktor, 2007), and the increased synthesis of other proteins. Persistent activation of PKMζ is also known to be involved in late LTP maintenance (Serrano, Yao, & Sacktor, 2005). Finally, late LTP is expressed via the addition of dendritic spines (i.e. the addition of new synapses) and an increase in post-synaptic surface area and sensitivity to glutamate. These changes are suggested to involve calpain, a protease activated by ERK that breaks down elements of the actin cytoskeleton, which would allow the synaptic reorganisation described above (Baudry et al., 2011). Late LTP expression translates behaviourally as the storage of a memory trace in long-term memory. Note that a consideration of this process is pertinent to the issue of memory reconsolidation, which will be discussed later in this chapter.

Indeed, several behavioural studies have lent support to the idea of LTP as the underlying neurophysiological mechanism for certain types of memory. Morris et al. (1986) trained rats in multiple trials over several days to locate and swim to a platform hidden beneath cloudy water. It was shown that rats that had been chronically administered the NMDA receptor antagonist 2-amino-5-phosphonovaleric acid (AP5) took significantly longer to find the platform than did rats in the control group. With a separate group of rats, Morris et al. (1986) were then able to demonstrate that AP5 blocked the induction of LTP in the hippocampus. The researchers concluded that their findings pointed to hippocampal NMDA receptor involvement in spatial memory. However, it is important to note that studies such as that of Morris et al. (1986) do not *directly* implicate the process of LTP in memory formation.

On the other hand, Whitlock et al. (2006) have provided some of the strongest direct evidence to date for the involvement of LTP in learning. Whitlock et al. (2006) trained rats over a single trial to avoid a darkened chamber and remain in an illuminated chamber via the delivery of a foot shock. Memory was measured as the avoidance of the darkened chamber in later trials. Rat hippocampi were then dissected and assayed, and it was observed that post-synaptic AMPA receptors were phosphorylated in the trained rats, but not in the untrained controls. Phosphorylation was also not observed when an NMDA receptor antagonist was applied. Furthermore, following training, increases in the number of AMPA receptors and in the size of the EPSP were observed. Note that these effects were observed in the hippocampus but not the cerebellum; hence, they were concluded to be region-specific rather than widespread. These are the same physiological events that result from high-frequency stimulation of the pre-synaptic neuron, and are some of the defining features of LTP (Sweatt, 1999). Whilst evidence for the induction of LTP following high-frequency stimulation abounds, no research prior to their study had directly examined whether LTP was induced in association with learning. Thus, Whitlock

et al.'s (2006) study has made an important link between the neurophysiological and behavioural aspects of memory formation.

Piracetam: An AMPAkine Drug

"AMPAkine" is the name given to a class of drugs that act at AMPA receptors as positive allosteric modulators; that is, they bind at an allosteric site on the AMPA receptor complex (i.e. a binding site that is not the active site) in order to produce their effect (for a review, see G. Lynch, 2006). Examples include aniracetam, BDP, cyclothiazide, CX516, CX546, IDRA-21, oxiracetam and piracetam. Given what has been described of LTP in the previous section – namely, that LTP involves the strengthening of synaptic connections as a result of increases in AMPA receptor numbers and activity – it is not surprising that a substance that facilitates AMPA receptor activity also facilitates LTP and memory function.

Some of the physiological effects attributed to AMPAkines in general include slowing of the desensitisation and deactivation of AMPA receptors and reducing the amount of afferent activity needed to induce LTP via the enhancement of AMPA receptor currents (G. Lynch, 2002, 2006; G. Lynch & Gall, 2006). It has also been suggested that AMPAkines may be effective in promoting LTP via increasing levels of endogenous BDNF (G. Lynch, Rex, Chen, & Gall, 2008; O'Neill, Bleakman, Zimmerman, & Nisenbaum, 2004). This is said to be achieved via an increase in synaptic activity, which has been shown to result in an elevation of BDNF synthesis. Note that the role of BDNF in memory will be further explored in a later section of this chapter.

The AMPAkine piracetam was the first nootropic drug to be developed (Giurgea, Lefevre, Lescrenier, & David-Remacle, 1971). With regard to cellular processes, piracetam has been shown to enhance LTP in the CA3 region of guinea pig hippocampal slices, but not the CA1 region (Satoh, Ishihara, & Katsuki, 1988, 1989; Sugimura, Ishihara, Katsuki, & Satoh, 1989). It has also been demonstrated to rescue induction of

LTP compromised by cerebral hypoperfusion in perforant path-CA3 synapses in rats (He, Liao, Zheng, Zeng, & Guo, 2008). Additionally, piracetam failed to affect LTP in dentate gyrus cells of rat hippocampi *in vivo* (Molnár & Gaál, 1992) and failed to rescue CA1 LTP impaired by alcohol exposure in rats (Chepkova et al., 1995). These results indicate that piracetam positively influences LTP, and furthermore suggest that piracetam's effect upon LTP is region-specific.

Piracetam's mechanism of action is a source of ongoing debate, as it appears to interact with several different neurotransmitter systems. While piracetam has low affinity for glutamate receptors (Bering & Müller, 1985), it has been demonstrated to be a weak modulator of AMPA receptors (Ahmed & Oswald, 2010; Copani et al., 1992) and has been shown to restore age-related deficits of NMDA receptors in aged mice such as reduced receptor density (Cohen & Müller, 1993). The memory-enhancing action of this drug can be inhibited by NMDA receptor antagonist MK-801 and cholinergic antagonist scopolamine (Sharma & Kulkarni, 1992). Stoll, Schubert and Müller (1991) have shown facilitation of muscarinic acetylcholine receptor function by piracetam in aged mice. Additionally, Mondadori and colleagues (Mondadori & Häusler, 1990; Mondadori & Petschke, 1987) have determined that piracetam's facilitation of memory in mice is dependent upon the function of aldosterone (mineralocorticoid) receptors, and Loscertales, Rose, Daisley and Sandi (1998) noted that intracerebral administration of both mineralocorticoid and glucocorticoid receptor antagonists interfered with piracetam's memory-enhancing action in the day-old chick.

A review by Gouliaev and Senning (1994) concluded that piracetam may exert its clinical effects via modulation of ion channels, which may potentially include the ion channel of AMPA receptors, which is in accordance with piracetam's known affinity for AMPA receptors. In contrast, Winblad (2005) suggests that piracetam acts via the restoration of cell membrane fluidity, and argues that this hypothesis accounts for the

drug's wide range of clinical applications. Indeed, a number of studies have demonstrated this effect upon neuronal membranes *in vitro*, particularly in aged brains and under circumstances of compromised fluidity (e.g. Eckert, Cairns, & Müller, 1999; Müller, Koch, Scheuer, Rostock, & Bartsch, 1997). It should be noted however, that these proposed mechanisms may not necessarily be mutually exclusive – there may be a common upstream mechanism that explains several of the observations described above (e.g. improvement of cell membrane fluidity may lead to the enhanced function of receptors and ion channels), or it may be that piracetam has multiple modes of action.

Effective facilitation of memory function by piracetam has also been demonstrated in humans. For example, Barnas et al. (1990) investigated the effect of a high dose of piracetam on cognitively impaired, chronic alcoholic participants. Using a double-blind design, they showed improvement in cognition specific to accuracy-based (rather than speed-based) tasks, including a memory measure. Piracetam has been investigated for the treatment of senile cognitive impairment; however, results regarding efficacy have been ambiguous (Vernon & Sorkin, 1991). Piracetam has also been shown to improve learning and memory in chronic schizophrenia (Dimond, Scammell, Pryce, Huws, & Gray, 1979). More recently, there has been evidence to suggest that piracetam may be useful in the treatment of the sequelae of closed head injury, including memory impairment (Zavadenko & Guzilova, 2009). As well as its effect in situations of memory compromise, piracetam has also been shown to be effective in improving memory in healthy participants. Dimond and Brouwers (1976) noted that undergraduate students administered piracetam daily over 14 days performed better than a placebo-administered control group when measured on a task of verbal memory.

Thus, piracetam can enhance memory in healthy experimental animals and normally-functioning human participants, as well as rescue memory impaired by a range of conditions. Piracetam has been demonstrated to facilitate LTP in the hippocampus in a

region-specific manner. Finally, while there is debate about piracetam's mechanism of action, it may be that the hypotheses put forward to date are not mutually exclusive, and may allow for multiple actions of the drug.

Memantine: An NMDA Receptor Antagonist

Memantine, a drug currently used in the treatment of Alzheimer's disease (AD), is a substance that acts as an uncompetitive NMDA receptor antagonist. That is, it blocks the action of NMDA receptor function, but not in competition with endogenous agonists. Its mechanism of *therapeutic* action is as yet not entirely understood, but it has nonetheless been shown to have a facilitatory effect on memory under conditions of memory compromise. Memantine has been demonstrated to improve cognitive function in moderate to severe AD and vascular dementia in human clinical trials (Orgogozo, Rigaud, Stöffler, Möbius, & Forette, 2002; Peskind et al., 2006; Winblad, Jones, Wirth, Stöffler, & Möbius, 2007; Winblad & Poritis, 1999). However, a recent meta-analysis suggests that memantine has limited effectiveness in mild cases of AD (Schneider, Dagerman, Higgins, & McShane, 2011).

Memantine has also been shown to rescue memory impairment in experimental animals. For example, memantine has improved spatial memory (Beracochea, Boucard, Trocme-Thibierge, & Morain, 2008) and recognition memory (Pietá Dias et al., 2007) in aged rats determined to have poorer memory relative to younger rats. Memantine has been demonstrated to ameliorate the impairment of memory task performance produced in rats by amyloid-β protein (Klyubin et al., 2011; Nyakas, Granic, Halmy, Banerjee, & Luiten, 2011; Yamada et al., 2005) and lipopolysaccharide-induced neuroinflammation (Rosi et al., 2006). In the day-old chick, memantine has also been demonstrated to rescue memory function impaired by isolation-induced stress (Barber, Meyers, & McGettigan, 2010).

Moreover, memantine has been shown to rescue deficits in LTP. Klyubin et al. (2011) reported that memantine, at a dose that partially impaired LTP, partially alleviated impairment of LTP by amyloid-β protein. A lower dose of memantine that did not disrupt LTP also alleviated amyloid-β protein's impairment of LTP. Additionally, memantine has been shown to restore LTP induction in hippocampal slices following the over-activation of NMDA receptors achieved via reduction of Mg^{2+} (Frankiewicz & Parsons, 1999).

As mentioned above, memantine acts as an antagonist at the NMDA receptor, and this receptor has been identified as the target of memantine's therapeutic mechanism. Interestingly, the notion that memantine's enhancement of memory function may involve action at NMDA receptors presents something of a paradox. NMDA receptor activation has been shown to be required for memory function (e.g. Morris et al., 1986; Whitlock et al., 2006), and yet memantine *inhibits* the function of this receptor. Parsons, Stöffler and Danysz (2007) have presented a possible explanation for this apparent contradiction. They suggest that under conditions of memory compromise, memantine dose-dependently restores homeostasis in the glutamatergic neurotransmitter system. They argue that over-stimulation of NMDA receptors is just as injurious to memory as is antagonism, and that the pharmacological properties of memantine allow it to selectively inhibit this pathological stimulation of NMDA receptor function while leaving physiological activation unaffected. This mechanism of action may account for the greater efficacy of memantine on patients suffering with severe AD relative to patients with mild AD as described earlier in this section – that is, memantine may be more effective under circumstances of severe memory disturbance because NMDA receptor over-stimulation may be more prevalent and thus present more of a target for action.

While there is a general consensus that the primary therapeutic mechanism for memantine involves the NMDA receptor (see Parsons et al., 2007; Rammes, Danysz, & Parsons, 2008; Rogawski & Wenk, 2003, for reviews), there is also some evidence to

suggest an interaction of memantine with cholinergic neurotransmission. According to Drever et al. (2007), the application of muscarinic acetylcholine receptor antagonist scopolamine impedes memantine's enhancement of synaptic transmission in the mouse hippocampus. Additionally, memantine has been shown to facilitate passive avoidance learning compromised by scopolamine in the day-old chick (Barber & Haggarty, 2010). These results do not necessarily directly imply an action of memantine at muscarinic receptors, but may suggest an interaction with processes further downstream.

The effect of memantine upon intact memory has also been examined; however, there has been considerably less investigation in this area relative to the study of memantine under circumstances of memory compromise. With respect to memory and cognitive performance in humans, a recent review has indicated that the acute application of memantine in healthy people has failed to lead to facilitation (Repantis, Laisney, & Heuser, 2010). The review noted that various studies have shown either no effect upon or impairment of memory and other cognitive functions such as attention (e.g. Rammsayer, 2001). Repantis et al. (2010) observed that there are no studies to date that have examined the effect of chronic memantine administration in healthy humans.

With regard to animal studies, findings are mixed. Some research is in accord with the abovementioned findings in humans; memantine has been demonstrated to have either a null or negative effect upon memory task performance in rats (Réus et al., 2008) and day-old chicks (Barber et al., 2010), and to impair induction of LTP (Klyubin et al., 2011). On the other hand, there has been some evidence to suggest that in healthy animals, memantine can enhance memory (Wise & Lichtman, 2007; Zoladz et al., 2006) and improve the maintenance of hippocampal LTP (Barnes, Danysz, & Parsons, 1996).

However, while there appears to be some evidence in support of the notion of memantine's nootropic action in healthy animals, this is inconsistent with the mechanism of action of memantine described by Parsons et al. (2007). Recall that these authors

suggest that memantine works via reducing pathological overstimulation of NMDA receptors to the exclusion of physiological activity – under the circumstance of intact functioning, there is no pathological overstimulation upon which memantine may act. Since Parsons et al. (2007) suggests that memantine does not affect normal NMDA receptor function, healthy memory function should not be affected by the application of memantine. How, then can one account for the reports of facilitation of healthy memory by memantine? Zoladz et al. (2006) provides a possible explanation for this observation. While these researchers found that memantine was effective in enhancing memory in healthy rats, they suggest that the nature of the memory task employed conferred a degree of stress upon the rats (i.e. immersion in cold water for a water maze task), and so memantine may actually have rescued memory impinged upon by stress. This may account for other findings in healthy animals, and may explain the overall smaller amount of evidence for memantine's enhancement of healthy memory and LTP function relative to that for its facilitation of impaired memory.

In sum, there appears to be strong evidence for memantine's efficacy in memory enhancement, particularly under circumstances of memory compromise. The evidence for improvement of healthy memory by the agent, however, is less conclusive. Memantine is suggested to act via modulation of NMDA receptor function, but it has also been implicated in cholinergic neurotransmission.

Brain-Derived Neurotrophic Factor and Memory

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family of endogenous growth factors, which bind to the tropomyosin-related kinase (Trk) family of receptor tyrosine kinases. BDNF binds specifically to the TrkB receptor (Barbacid, 1994). Neurotrophins have a well documented role in neuronal survival and differentiation (e.g. Snider, 1994), yet recent research suggests that BDNF also has a function in activity-dependent synaptic plasticity (for reviews, see B. Lu, 2003;

McAllister et al., 1999; Poo, 2001). As described earlier in this chapter, an example of synaptic plasticity is LTP, and since LTP is believed to underlie memory function (e.g. Morris et al., 1986; Whitlock et al., 2006), it is not surprising that a role for BDNF has also been demonstrated in memory (Tyler, Alonso, et al., 2002; Yamada et al., 2002).

With regard to synaptic plasticity, there is abundant evidence to support a role for BDNF. Figurov, Pozzo-Miller, Olafsson, Wang and Lu (1996) have demonstrated that in the rat hippocampus *in vitro*, the application of exogenous BDNF enhances LTP induced by tetanic stimulation. Significant increases in levels of BDNF mRNA have been found following induction of LTP in the rat hippocampus (Patterson, Grover, Schwartzkroin, & Bothwell, 1992). Blockade of TrkB receptors, to which BDNF binds, results in impaired late LTP (Korte, Kang, Bonhoeffer, & Schuman, 1998). Furthermore, LTP is demonstrably impaired in BDNF-deficient animals (Korte et al., 1995), and it has been shown that the administration of exogenous BDNF to the deficient animals reverses the impairment (Patterson et al., 1996). These findings clearly implicate endogenous BDNF in LTP, and there is now widespread acceptance of this notion (Cunha, Brambilla, & Thomas, 2010; B. Lu, 2003; McAllister et al., 1999; Minichiello, 2009; Poo, 2001).

Further detail regarding the possible mechanism of BDNF's action in LTP has been described in recent reviews (Cunha et al., 2010; Minichiello, 2009), although definitive support remains elusive. BDNF acts by binding to TrkB receptors as mentioned above, initiating an intracellular signalling cascade. Specifically, this may involve the activation of phospholipase Cγ (PLCγ; Gärtner et al., 2006; Minichiello et al., 2002), which in turn leads to the release of Ca^{2+} from intracellular stores. As described in an earlier section of this chapter, activation of kinases (e.g. CAMKII) by Ca^{2+} is integral to early LTP. Initiation of the MAPK/ERK cascade may also be involved in BDNF's action in LTP (Ying et al., 2002). This is consistent with Baudry et al.'s (2011) conception of LTP in which BDNF leads to activation of calpain via a MAPK mechanism

(Zadran et al., 2010). Calpain has been suggested to have a role in structural reorganisation of the neuron; that is, expression of LTP. BDNF/TrkB activity, via a tyrosine kinase called Fyn, leads to the phosphorylation and enhanced function of NMDA receptors (Mizuno, Yamada, He, Nakajima, & Nabeshima, 2003). Lastly, BDNF is believed to have presynaptic functionality in LTP in addition to the postsynaptic functions described above, such as the modulation of vesicular release of neurotransmitters (Tyler, Perrett, & Pozzo-Miller, 2002).

This discussion leads to the question of whether the previously described cellular events correspond to a role for BDNF in memory as demonstrated in behavioural studies. Indeed, this has been shown to be the case (Yamada et al., 2002). Impairment of performance on memory tasks has been demonstrated in experimental animals following injection of BDNF antibodies (Alonso et al., 2002; Johnston, Clements, & Rose, 1999), administration of BDNF antisense oligodeoxynucleotides (ODN; Johnston & Rose, 2001; J. L. C. Lee, Everitt, & Thomas, 2004) and deletion of the BDNF gene (Heldt, Stanek, Chhatwal, & Ressler, 2007). BDNF's role in memory is also indicated by the increase in BDNF mRNA expression following learning in rats noted by Alonso et al. (2002). BDNF may also be involved in the persistence of long-term memories, as demonstrated by Bekinschtein and colleagues (Bekinschtein et al., 2007; Bekinschtein et al., 2008). Behavioural data have also corroborated the role of the MAPK/ERK pathway in BDNF's function in memory (Bekinschtein et al., 2008; McGauran et al., 2008). Johnston et al. (1999) and Alonso et al. (2002) noted improved performance on memory tasks after animals were injected with exogenous BDNF, although Cirulli et al. (2000) found no effect of injected BDNF on memory performance in rats. Fischer, Sirevaag, Wiegand, Lindsay and Björklund (1994) also demonstrated a lack of effect for BDNF upon memory function. They noted that BDNF failed to rescue compromised spatial memory in aged rats, while other neurotrophins were noted to do so. Despite some negative

findings, it is now generally accepted that BDNF does indeed play an important role in memory processing (Y. Lu, Christian, & Lu, 2008; Yamada et al., 2002).

Taken together, these findings indicate that BDNF plays a role in both the process of LTP and of memory consolidation. Some of these results further indicate that BDNF is in fact necessary for consolidation of some types of memory. The mechanism behind BDNF's role in these processes has not been well characterised as yet, but there are strong indications that the PLCγ pathway and the MAPK/ERK cascade are involved.

Memory Reconsolidation

It has been argued that when a previously consolidated memory is recalled from long-term storage, it once again becomes susceptible to change. The memory trace, once retrieved, is able to be modified in some way and then *reconsolidated* (Nader & Einarsson, 2010; Nader & Hardt, 2009; Tronson & Taylor, 2007). This proposition makes intuitive sense and is also supported empirically. When taking in new information, it is reasonable to expect that some level of integration of the new information with existing knowledge would take place. For example, in order to memorise the name of a new acquaintance, one might associate the new name with the name of the friend that had introduced you. Thus, you are able to recall the name of the new acquaintance after making the association between the acquaintance and your mutual friend. Presumably, some modification of the memory trace of the name of the mutual friend must have taken place in this process, followed by reconsolidation of the modified and updated memory.

Indeed, research has demonstrated such modification in the form of impaired memory for a well-learned task following administration of MK-801, a drug known to interfere with memory, in association with a "reminder" trial (Przybyslawski & Sara, 1997). The study involved over-training rats on an eight-arm radial maze task. The rats were injected with either MK-801 (an NMDA receptor antagonist) or saline following a single training trial (in this thesis, this is referred to as a reminder trial, although

Przybyslawski & Sara, 1997, use the term “reminder” differently in their study), and were then tested on the maze task 24 hours later. It was found that injection of MK-801 up until around 90 min post-reminder trial led to significantly greater errors relative to the saline control condition on delayed test trial performance. When a more difficult version of the maze task was implemented, the deficit resulting from MK-801 administration was found when the injection was delivered up to 3 hours after the reminder trial. Further experimentation revealed that a single session of multiple training trials was sufficient to retrain the rats to perform the maze task, and that a decrement in performance resulting from administration of MK-801 was observed even when the test trial was given 48 hours after the injection, ruling out the possibility of spontaneous recovery from the amnesia induced by MK-801. Przybyslawski and Sara (1997) concluded that the reactivation of the previously consolidated memory traces by the reminder trial rendered them labile and able to be modified by the administration of MK-801. Similar findings have been reported in rats with MK-801 in an appetitive learning task (J. L. C. Lee & Everitt, 2008) and with AP5 (another NMDA receptor antagonist) in an odour discrimination task (Torras-Garcia, Lelong, Tronel, & Sara, 2005).

Findings implicating NMDA receptor activity in reconsolidation have also been noted in the day-old chick. In a study carried out by Summers, Crowe and Ng (1997), chicks were trained on the PAL task, and the NMDA receptor antagonist AP5 was administered at the time of a reminder trial. When the reminder trial was presented at various times up to 24 hours post-training, memory deficits were seen upon testing 60 min post-reminder. Furthermore, the period of time post-reminder over which AP5 led to poor recall decreased as the time between training and reminder increased. This transient deficit was noted to resolve by 24 hours in all cases. When the reminder was given 48 hours after learning, no deficit resulting from AP5 administration was seen.

Thus, blockade of NMDA receptor function by AP5 at the time of a reminder leads to transient impairment of recall in the day-old chick.

Further evidence for the role of the NMDA receptor in reconsolidation has been established using memantine. This drug has been observed to have an inhibitory effect upon memory reconsolidation. Popik, Wrobel and Bisaga (2006) demonstrated that in rats, memantine prevents the reinstatement of morphine-conditioned place preference primed by morphine, when it is injected at the time of a reminder. In other words, despite priming by morphine, the rats were not able to recall the memory of place preference due to memantine's interference with reconsolidation of the memory. This finding is in keeping with memantine's antagonistic effect at NMDA receptors and with the positive role of NMDA receptors in the process of reconsolidation described above. However, it is inconsistent with memantine's facilitatory role in memory described earlier in this chapter. This difference may be accounted for by variations in dose, an issue that is discussed in further detail in Chapter 3.

To date there has been relatively little research with regard to the involvement of AMPA receptors in reconsolidation. One study has reported that while NMDA receptors are involved in the *induction of lability* of an auditory fear memory and not the expression of the memory, the reverse was noted to be true for AMPA receptors (Mamou, Gamache, & Nader, 2006). These researchers found that at 24 hours post-training, infusion of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; an AMPA receptor antagonist) in the basolateral amygdala prior to a reminder trial was not able to prevent anisomycin-induced amnesia for the task upon testing a further 24 hours later. If activation of AMPA receptors was necessary for the induction of lability of a memory trace in this task, blockade would be expected to lead to intact memory at test, so Mamou et al.'s (2006) findings were taken to indicate that AMPA receptors were not required in this process. On the other hand, CNQX infusion led to poor memory recall during the reminder trial,

suggesting that AMPA receptors are necessary for the expression of the memory at the time of memory reactivation.

However, the question of whether or not AMPA receptors play a role in the *reconsolidation* of the memory after it has been rendered labile persists. Recent research involving the day-old chick has begun to answer this question. Chicks trained on the PAL task were given a reminder at 120 min post-learning, and application of CNQX 20 min after presentation of the reminder led to impaired recall upon testing from 90 to 180 min post-reminder, but not earlier (Sherry & Crowe, 2008). As in the study by Summers et al. (1997), the deficit was transient in nature – it was observed to resolve by 24 hours post-reminder. This finding suggests a role for AMPA receptors in the later stages of reconsolidation. Nevertheless, since there is only limited evidence for the involvement of AMPA receptors in reconsolidation thus far, further study is warranted. AMPAkines such as piracetam may be useful in further investigation, although this drug has not previously been used in the study of reconsolidation.

Recently, evidence employing BDNF has emerged in support of the notion that reconsolidation is not equivalent to consolidation and is in fact a distinct process. A landmark study by J. L. C. Lee et al. (2004) demonstrated a double dissociation in the factors involved in memory consolidation and reconsolidation in the rat hippocampus. Rat dorsal hippocampi were infused with antisense ODNs, which inhibit the synthesis of specific proteins, and the rats were trained on a contextual fear conditioning task. Consolidation was shown to involve BDNF but not transcription factor Zif268, given that infusion with BDNF antisense ODN 90 min before fear conditioning led to a disruption in long-term memory (LTM) associated with the conditioning relative to control, whereas infusion with Zif268 antisense ODN did not. It should be noted that short-term memory remained intact in both cases. On the other hand, reconsolidation was demonstrated to require Zif268 but not BDNF. Twenty-four hours after conditioning, a reminder trial was

administered and a further 24 hours later, fear conditioning retention was measured. Infusion with Zif268 antisense ODN 90 min before the reminder trial led to a disruption in LTM for the fear conditioning but did not do so at the time of the reminder trial. Importantly, no deficit in LTM was found when the reminder trial was not administered, which further supports the idea that reactivation of memory traces renders them once again susceptible to modification. Finally, no effect on reconsolidation was found as a result of infusion of BDNF antisense ODN relative to control. J. L. C. Lee et al.'s (2004) findings lend considerable support to the view that memory consolidation and reconsolidation are indeed distinct processes. Moreover, other dissociations like that of J. L. C. Lee et al. (2004) have also been noted in the day-old chick. For example, ouabain (a Na^+/K^+-ATPase inhibitor) has been shown to impair memory consolidation (Gibbs & Ng, 1976) but not reconsolidation (Sherry & Crowe, 2007).

Thus far we have seen strong evidence to uphold both the notion of reconsolidation and its distinction from consolidation. Recent reviews discuss further evidence along a similar vein (Nader & Einarsson, 2010; Nader & Hardt, 2009; Tronson & Taylor, 2007). It is also reasonably well accepted that reconsolidation is a partial recapitulation of consolidation – in other words, reconsolidation may involve some of the same processes of consolidation, but is it not an identical process (Alberini, 2005). However, there is ongoing debate regarding the specification of this process. While several hypotheses have been put forward, a review by Tronson and Taylor (2007) concludes that most evidence points toward the notion that reconsolidation is a mechanism responsible for storage of existing memories that have been retrieved, and that this mechanism is independent of initial consolidation. Although some findings indicate that deficits in reconsolidation following memory reactivation are transient, particularly in the chick (e.g. Anokhin, Tiunova, & Rose, 2002; Sherry & Crowe, 2008; Summers et al., 1997), and that post-reminder disruption (and therefore disruption of

reconsolidation) may only occur within a certain period post-training (Crowe, Sherry, & Hale, 2008), Tronson and Taylor (2007) point out that this time period of possible disruption may vary with respect to the strength of the reminder. These authors also note that, given the difficulty in experimentally separating memory retrieval and storage mechanisms, it is unclear whether observed reconsolidation deficits are due to retrieval deficits (cf. Anokhin et al., 2002) or changes to the underlying memory trace (cf. J. K. Rose & Rankin, 2006).

It is important to be aware of the limiting conditions of reconsolidation. Among the large number of studies that have investigated reconsolidation, there are wide varieties of learning tasks employed (and therefore, types of memory involved), and brain structures and molecular mechanisms targeted. The fact that reconsolidation has been confirmed across such a wide range of tasks supports the existence of the process, but it has not been found to occur in all tasks studied. For example, one study reported no disruption following memory reactivation in rats trained on a step-down inhibitory avoidance task, and concluded that there was no evidence for reconsolidation of this task (Cammarota, Bevilaqua, Medina, & Izquierdo, 2004). Other limiting factors may include the age of the memory (i.e. the time since training), the duration of application of the amnestic agent and, as mentioned above, the strength of the reminder (Tronson & Taylor, 2007).

Thus, while the mechanism of reconsolidation is as yet not fully understood and a universal conceptualisation has not been agreed upon, it is well established that the reconsolidation process exists and that the process is similar yet distinct from consolidation. Reconsolidation appears to involve NMDA receptor activity, and initial data support an important role for AMPA receptors but not for BDNF. Finally, limited research has investigated the impact of memantine on reconsolidation and there is no

evidence to date of the effect of piracetam. Studies involving these drugs may assist in outlining the mechanisms involved in reconsolidation.

The Day-Old Chick and the PAL Task

Neonate domestic chicks have been widely used in memory research. They are precocial animals with well-developed brains and they actively explore and learn about their environments from very early on in life. Young chicks also have soft skulls, and at this early stage in life, their blood-brain barrier is not yet fully developed. These attributes are advantageous as they allow easy administration of pharmacological agents (Andrew, 1991; Gibbs & Ng, 1977; S. P. R. Rose, 2000).

In the current studies, the day-old domestic chick will be employed in conjunction with the PAL task, introduced by Cherkin (1969). The task involves training chicks to avoid pecking at an aversive-tasting bead in a single trial, and has the advantages of temporal precision with respect to the learning event and measurement of memory retention and reliance of the dependent measure on a behavioural event natural to the chick's repertoire – that is, the peck response (Andrew, 1991).

Since its introduction, different laboratories around the world have used different variants of the PAL task. The current adaptation of the task involves pretraining with a water-coated chrome bead, a water-coated red bead as a baseline measure, training with a red bead dipped in 20% v/v methyl anthranilate (MeA; the aversant), and testing with a visually identical, dry red bead (e.g. Crowe & Hale, 2002, 2004). In studies of memory reconsolidation, a dry red bead is presented sometime after training as a "reminder", in order to induce recall of the training event. In order to investigate the effects of a substance on memory formation, injection of the substance may be carried out at various time points relative to the training trial, or to other stages in the task. Note that other laboratories have used different bead colours and sizes, different concentrations of MeA (100% v/v is considered "strong" training, whilst 20% v/v is considered "weak" training)

and different timing of the task phases (for more detailed discussions of procedural variations, see Crowe & Hamalainen, 2001; Gibbs, Johnston, Mileusnic, & Crowe, 2008).

Furthermore, different dependent measures have been used in the PAL task, although all relate to whether or not the chick pecks at the bead during the test phase. One common measure involves calculation of an "avoidance ratio", which relates the number of pecks to the test bead to the number of pecks to the baseline bead (e.g. Crowe & Hale, 2002, 2004). A "discrimination ratio" has also been used, which relates the number of pecks to the test bead to the number of pecks to a subsequently presented bead of another colour (e.g. Gibbs & Ng, 1984). Both ratios are continuous in nature (allowing parametric statistical analysis) and have a numerical range of 0 to 1, where 1 indicates complete avoidance of the test bead. A value of 0.5 indicates equal pecking to the test and baseline/discrimination beads. In the current research, the dependent variable is a binary measure – no pecks to the test bead vs. one or more pecks, and non-parametric analyses have been employed. This measure and associated statistical analyses were chosen due to the fact that initial continuous data collected were shown to violate both the assumptions of normality and of homogeneity of variances of parametric analyses (Tabachnick & Fidell, 2007).

Results of the weak vs. strong versions of the PAL task have also differed across laboratories. In the absence of memory-influencing factors (e.g. memory-enhancing drugs, stress), chicks tested in this laboratory have been demonstrated to retain memory for the strong training paradigm for up to 24 hours post-training, whereas memory for the weak training paradigm (using 20% MeA v/v) lasts around 35-45 min post-training (Crowe & Hale, 2002, 2004; Crowe, Ng, & Gibbs, 1989). Rose and colleagues have found similar results for the strong training paradigm; however, these researchers have found that weakly reinforced learning (using 10% MeA v/v) leads to a memory trace

lasting several hours (e.g. Sandi & Rose, 1994). It is not clear what has led to such a discrepancy, although one proposed suggestion is that protocol differences between the laboratories have led to a difference in the salience of the training experience (Gibbs et al., 2008). This would account for the different lengths of time over which memory traces have been observed to last, given that strength of the training event has been shown to impact upon the endurance of a memory trace (Crowe et al., 1989). Despite these differences, it has been observed that findings are generally well able to be replicated across laboratories.

In this laboratory, the PAL task has led to the development of an experimental model of memory as proposed by Gibbs and Ng using pharmacological and behavioural data (Gibbs & Ng, 1976, 1977, 1984; Ng et al., 1997). This model is comprised of three phases and described as being sequentially-dependent – that is, formation of a later phase of memory depends on the intact formation of all preceding phases. The three phases are as follows:

(1) Short-term memory (STM): lasts until around 10 min after the learning event, but may take until around 30 min post-learning to decay completely. Evidence for STM has been presented by Gibbs and Ng (1976). They demonstrated that administration of lithium chloride or potassium chloride inhibited memory in chicks when tested immediately after training and onward. However, administration of ouabain prior to learning leads to amnesia only after 10 min following learning (i.e. from 15 min post-learning onward), whereas memory up until this time was observed to be intact.

(2) Intermediate memory (ITM): also called labile memory, begins at around 20 min and decays from about 50 min post-learning. This phase is divided into two sub-phases termed ITM(A) and ITM(B). The end of ITM(A)/start of ITM(B) is at around 30 min after learning. As mentioned above, Gibbs and

Ng (1976) have demonstrated that ouabain selectively impairs memory recall from 15 min post-learning onwards – this corresponds to impairment of ITM(A). The impairment by ouabain can be rescued by diphenylhydantoin, a substance that stimulates Na^+/K^+-ATPase activity, thus ITM(A) is said to be dependent on the action of Na^+/K^+-ATPase. Additionally, the two phases of ITM have been distinguished from each other by a double dissociation – ITM(A) is inhibited by 2,4-dinitrophenol but not by cycloheximide, whereas ITM(B) is inhibited by cycloheximide and not by 2,4-dinitrophenol (Gibbs & Ng, 1984).

(3) Long-term memory (LTM): begins at around 60 min post-learning and continues potentially indefinitely. LTM is said to be dependent upon protein synthesis, as it is impaired by protein synthesis inhibitors cycloheximide and anisomycin (Gibbs & Ng, 1977, 1984). LTM has also been demonstrated to be impaired by various PKA inhibitors (Zhao et al., 1995). Recall that PKA is a substance involved in the induction and maintenance of early LTP; thus, Zhao et al.'s (1995) results are consistent with disruption of LTP.

The three phases of memory formation are also separated by transient memory deficits at 15 and 55 min post-learning, which are interpreted within the model as being due to transitions between STM and ITM, and ITM and LTM, respectively (Gibbs & Ng, 1979). These deficits in recall are said to occur whilst one phase is decaying and before the subsequent phase has initiated.

The Gibbs and Ng model of memory in the day-old chick is particularly useful in the investigation of memory-enhancing drugs. It should be possible to align the effects of drug administration upon memory with a particular phase or phases in the model. Data about the mechanisms underlying each of the phases may then be used to suggest

mechanisms for the drugs. Therefore, it is this model that is used in the interpretation of findings of the current series of studies.

The Current Research

The general aim of the present series of studies was to investigate two memory-enhancing agents, piracetam and memantine, using the day-old chick as a model. As discussed earlier in this chapter, these two drugs are reported to have differing cellular mechanisms that are involved in LTP, a process widely believed to underpin memory formation. Therefore, piracetam and memantine were selected for their ability to positively modulate memory function in different ways, with a view to shedding light on underlying mechanisms of memory consolidation and reconsolidation. BDNF was also chosen for investigation of its memory-enhancing effect for the same reason, given the recent interest in this substance's role in synaptic plasticity and memory as outlined above. Specifically, this research addressed the following questions: (1) What is the most effective dose of each substance in facilitating memory? (2) For piracetam and memantine, in what time window does injection of the drug lead to significant memory enhancement at test? (3) Over what time period following learning does injection of each substance lead to enhanced memory recall? (4) What role, if any, does each substance have in memory reconsolidation?

This thesis is comprised of three studies that deal with each of the research questions for the three substances investigated. The methodology is identical for each of the studies, with the exception of the substance injected, and the studies have been carried out in parallel. Chapter 2 describes the series of experiments with piracetam, Chapter 3 describes the experiments with memantine and Chapter 4, the experiments with BDNF. Finally, in Chapter 5, a general discussion brings together the results of the three studies and integrates these findings with existing research on memory mechanisms involving piracetam, memantine and BDNF.

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Chapter 2: Piracetam Enhances Memory Consolidation and Reconsolidation in the Day-Old Chick

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Abstract

Piracetam is an AMPAkine drug that may have a range of different mechanisms at the cellular level, and which has been shown to facilitate memory, amongst its other effects. This series of experiments demonstrated that a 10 mg/kg dose of piracetam facilitated memory consolidation in the day-old chick when injected from immediately until 120 min after weak training (i.e. using a 20% v/v concentration of methyl anthranilate) using the passive avoidance learning task. Administration of piracetam immediately after training led to memory facilitation that lasted for up to 24 hours following training. This dose of the AMPAkine also facilitated recall at 180 min post-reminder when administered in conjunction with a reminder trial. These findings support the contention that application of the AMPAkine piracetam facilitates both consolidation and reconsolidation of memory using a weak training task, and extend the range of actions previously noted with NMDA-related agents to those that also facilitate the AMPA receptor.

Keywords: Piracetam Passive avoidance Day-old chick Consolidation Reconsolidation

Introduction

The AMPAkines are a group of agents that produce their effect by facilitating the action of α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors (for a review, see Lynch, 2002). Some examples of this class of drug include piracetam, aniracetam, BDP, CX516, CX546, IDRA-21 and cyclothiazide. AMPA receptors are involved in long-term potentiation (LTP), which is believed to be the key neurophysiological process underlying the formation and consolidation of memory (e.g. Lynch, 1998). It has been previously shown that inhibition of AMPA receptor activity results in memory deficit (e.g. Rickard, Poot, Gibbs, & Ng, 1994). The facilitative effect of various AMPAkines on memory functioning has been explored in both animals and humans. AMPAkines have been shown to facilitate both AMPA receptor activity (Hampson, Rogers, Lynch, & Deadwyler, 1998a; Ito, Tanabe, Kohda, & Sugiyama, 1990; Nagarajan, Quast, Boxall, Shahid, & Rosenmund, 2001; Stäubli, Rogers, & Lynch, 1994) as well as memory functioning (Buccafusco, Weiser, Winter, Klinder, & Terry Jr, 2004; Davis et al., 1997; Goff et al., 2007; Hampson, Rogers, Lynch, & Deadwyler, 1998b; Larson et al., 1995; Lynch et al., 1997; Stäubli, Perez, et al., 1994; Stäubli, Rogers, et al., 1994; Wezenberg, Jan Verkes, Ruigt, Hulstijn, & Sabbe, 2006).

Piracetam is an AMPAkine of particular interest as it appears to have a number of mechanisms of action. While some researchers have shown that piracetam works via positive modulation of AMPA receptors (Ahmed & Oswald, 2010; Copani et al., 1992), the drug has also been reported to act at a wide variety of other sites, although it appears to have very low or no affinity for any specific receptor type (Gouliaev & Senning, 1994). Some studies have reported facilitation of NMDA and acetylcholine receptor activity (Sharma & Kulkarni, 1992; Stoll, Schubert, & Müller, 1991). It has also been suggested that the action of piracetam occurs via the restoration of cell membrane fluidity (Winblad, 2005). Yet other studies have proposed that the facilitative action of

piracetam on memory function involves a brain corticosteroid-related mechanism (Loscertales, Rose, Daisley, & Sandi, 1998; Mondadori & Häusler, 1990). The comprehensive review prepared by Gouliaev and Senning (1994) proposes that piracetam exerts its various effects by acting on membrane ion channels.

Piracetam has been shown to improve memory function in human clinical trials. For example, Barnas et al. (1990) used a double-blind design to show that a relatively high dose of piracetam (1200 mg b.i.d.) led to improvement in cognition in chronic alcoholic participants who had been verified as cognitively impaired prior to the study. The improvement was specific to tasks (including the memory measure) that relied upon accuracy rather than speed as the outcome measure. Piracetam has also been shown to be effective as a memory-enhancing agent in healthy undergraduate students, with individuals administered 400 mg of piracetam daily performing better on a measure of verbal memory after 14 days than did the individuals administered placebo (Dimond & Brouwers, 1976).

An important issue in contemporary memory research relates to the issue of memory reconsolidation. It has been argued that when a previously consolidated memory is recalled from long-term storage, it once again becomes susceptible to change. The reactivated memory trace is then able to be modified and then *reconsolidated,* incorporating the new information (see Crowe, Sherry, & Hale, 2008; Tronson & Taylor, 2007, for reviews). When taking in new information, it is reasonable to expect that some level of integration of the new information with existing knowledge must take place. A study by Mamou, Gamache and Nader (2006) reported that NMDA receptors, but not AMPA receptors, are involved in changing a memory trace from a fixed to a labile state in order for reconsolidation to occur. AMPA receptor activity has, however, been implicated in later stages of reconsolidation in the day-old chick. For example, Sherry and Crowe (2008) have demonstrated that the application of CNQX (an AMPA receptor

antagonist) in association with the presentation of a reminder trial resulted in deficits in later retention. They noted that retention was not permanently impaired since the deficit was seen to resolve by 24 hours post-reminder, indicating that retrieval of the memory involved a different process to that involved in the initial consolidation of that memory (i.e. because the underlying memory trace was not affected).

The present research employed the day-old chick as subject and the passive avoidance learning (PAL) paradigm (Crowe & Hale, 2002), which has been used extensively in previous research (see Gibbs, Johnston, Mileusnic, & Crowe, 2008, for review). The PAL task exploits the chick's natural tendency to peck at bright objects, and has the advantage of temporal precision with respect to the learning event – chicks are exposed to an aversive bead for only a very brief period of time (i.e. 10 s) – thus enabling researchers to pinpoint the initiation of memory formation. The current research set out to characterise the effect of the AMPAkine piracetam on passive avoidance learning in the day-old chick by determining the most effective dose (Experiment 1), the most effective time to inject the drug (Experiment 2), the length of time over which piracetam enhances memory (Experiment 3) and the ability of piracetam to enhance memory in the context of reconsolidation (Experiment 4).

Method

Subjects. One thousand and eighty male day-old New Hampshire × White Leghorn chicks (*Gallus domesticus*; average weight of 45 g) served as subjects: 240 in Experiment 1, 320 each in Experiments 2 and 3, and 200 in Experiment 4. The chicks were housed in pairs (to reduce any distress brought about by social isolation) in open topped, wooden boxes (20 × 25 × 20 cm). One chick in each pair was marked on the head with a black felt tip pen for the purpose of identification during data collection. The chicks were kept warm by a 60 W incandescent light bulb suspended above each box, maintaining a temperature of 27-32°C, and chick mash was provided *ad libitum*. Water

was not provided. Each experimental condition consisted of a group of 20 chicks initially, but the final number varied depending on the number of chicks that successfully completed the baseline and training phases. Chicks were allowed to settle for at least 30 min prior to the onset of experimentation.

Materials. Piracetam (2-oxo-1-pyrrolidineacetamide) was injected subcutaneously in all four experiments. Each chick was injected with a volume of 0.1 mL using a 1 mL syringe with a 27 gauge needle. Saline solution was administered as the control substance. The PAL task required a chrome bead (2 mm in diameter) and two red glass beads (4 mm in diameter), each soldered to the end of a wire rod, and the aversive substance methyl anthranilate (MeA). Where MeA was used at a concentration of 20% v/v, the MeA was diluted with ethanol. The number of pecks for each chick in each trial was recorded using a handset connected to a PC in the laboratory so that button presses on the handset could be recorded and summed automatically.

Procedure and design. Each experiment employed the La Trobe University variant of the PAL task (see Crowe & Hale, 2002; Gibbs et al., 2008). Firstly, the chicks were presented with a water-coated chrome bead for two trials of approximately 10 s, spaced 30 min apart, to encourage the pecking response (chrome pretraining phase; P). Another 30 min later, a water-coated red bead was presented for 10 s and the number of pecks to the bead was recorded as a baseline measure (red baseline phase; B). A further 30 min later, a second, visually identical red bead was coated with the aversive substance MeA, then presented to the chicks for 10 s (training phase; Tr). In Experiment 4 only, following a delay, the chicks were presented with a dry red bead for 10 s but were not allowed to peck at it (reminder phase; R). The presentation of a ‘reminder’ of the previously consolidated memory had the purpose of rendering the memory trace labile and once again subject to possible alteration. Finally, after a period of delay, the chicks were exposed to the dry red bead and the number of pecks to this bead was recorded as a

measure of retention of the learning event (red test phase; Te). Injection of piracetam or saline solution occurred at various times relative to the training phase (Experiments 1-3) or to the reminder phase (Experiment 4). See Figure 2.1 for a summary of the experimental design of each of the four experiments. Chicks that did not peck at either the red baseline or training phases were excluded from the analysis (generally no more than 15% were excluded on this basis).

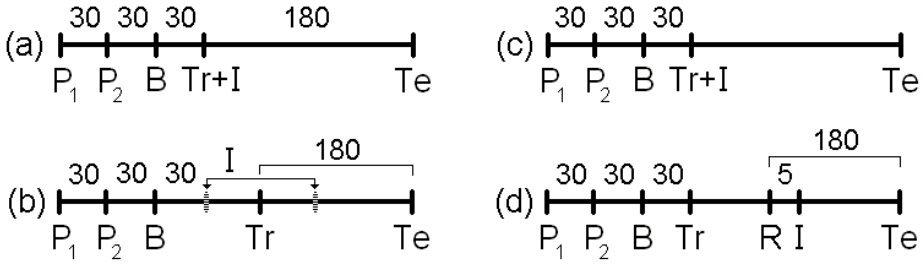

Figure 2.1. Timeline of events for (a) Experiment 1 (dose response), (b) Experiment 2 (time of injection), (c) Experiment 3 (time of test) and (d) Experiment 4 (time of reminder trial). Numbers above the lines indicate minutes between events marked on the lines. P = pretraining, B = baseline, Tr = training, I = injection, R = reminder, Te = test.

In Experiment 1, the independent variables (IVs) were concentration of MeA at training (20% vs. 100% v/v) and dose of piracetam (0, 5, 10, 25, 50 and 100 mg/kg). Piracetam was injected immediately following the training phase, and retention was measured at 180 min after the training phase.

In Experiment 2, the IVs were time of injection relative to the training phase (60, 15 and 5 min before, immediately, 5, 15, 60 and 120 min after) and drug injected (saline vs. 10 mg/kg piracetam – the most effective dose as determined in Experiment 1). The

concentration of MeA used was 20% v/v and retention was measured 180 min after training.

In Experiment 3, the IVs were time of test following the training phase (10, 20, 40, 60, 90, 120, 180 and 1440 min) and drug injected (saline vs. 10 mg/kg piracetam). The concentration of MeA used was 20% v/v and injection occurred immediately after the training phase.

In Experiment 4, the IVs were time of reminder trial relative to the training phase (10, 20, 40, 60 and 180 min) and drug injected (saline vs. 10 mg/kg piracetam). The concentration of MeA used was 20% v/v, injection occurred 5 min after the reminder trial and testing was carried out 180 min after the reminder trial.

Due to significant skewing of the originally calculated dependent measure for all experiments (an avoidance ratio), the dependent variable employed was a binary measure with the two levels coded as avoidance (i.e. no pecks to the red test bead, therefore demonstrating memory for the training event) or no avoidance (i.e. one or more pecks to the red test bead, therefore demonstrating a lack of memory for the training event).

Results

Experiment 1: Dose-response. Hierarchical logistic regression was carried out to investigate the impact of dose of piracetam and [MeA] (20% vs. 100% v/v) on whether or not chicks avoided the red test bead (with avoidance indicating memory for the training event). Main effects of dose and [MeA] were entered in the first step and the dose-by-[MeA] interaction term was entered in the second step. The interaction step was found to be significant ($\chi^2(5) = 16.164$, $p = 0.006$); therefore, simple main effects were examined using Pearson's chi-square tests ([MeA] by avoidance) at each dose. Figure 2.2a illustrates the percentage avoidance for each experimental group. Non-significant [MeA]-by-avoidance cross-tabulations at piracetam doses of 5, 10 and 25 mg/kg ($p > 0.05$) indicate effective facilitation of memory – that is, injection of

piracetam at these doses rendered the normally 'weak' training paradigm of 20% v/v MeA equivalent to the 'strong' 100% v/v MeA training paradigm. Given the inverted-U dose-response curve produced in this experiment, the 10 mg/kg dose of piracetam (being the median of the three effective doses) was subsequently employed for Experiments 2-4.

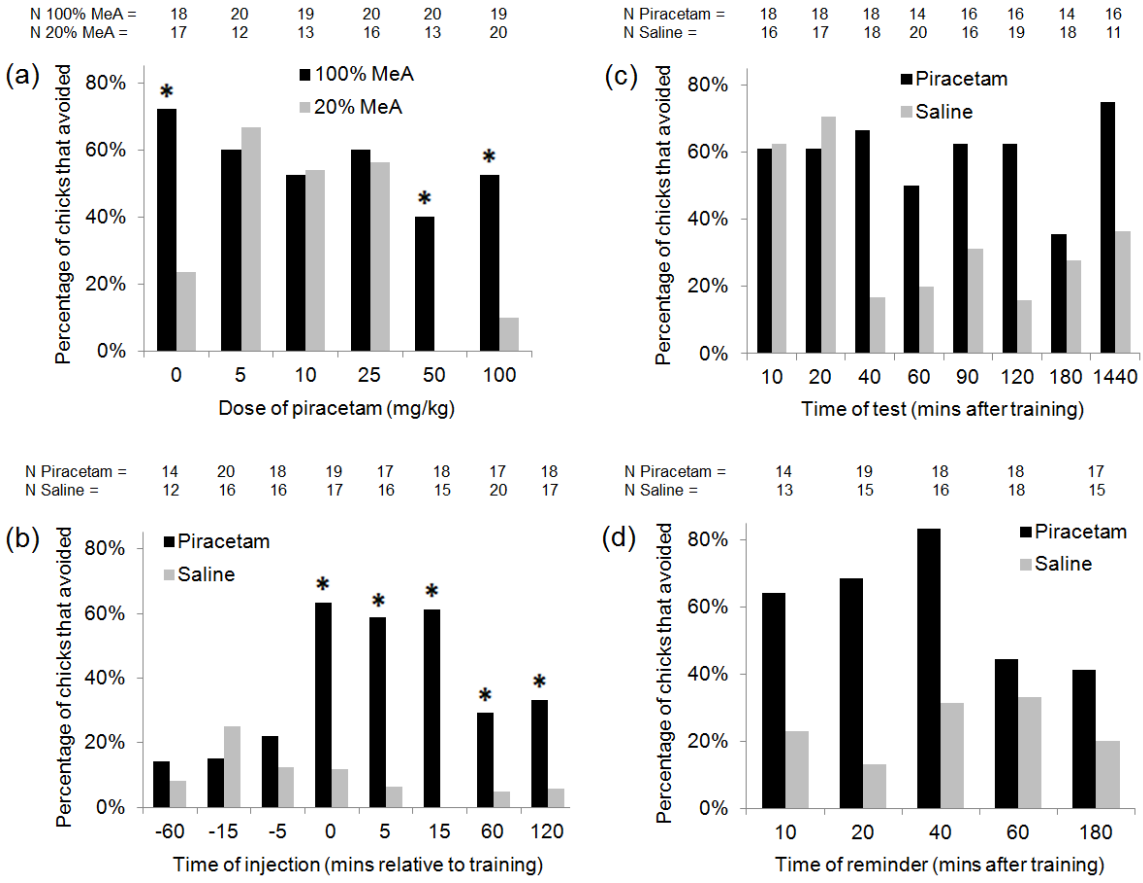

Figure 2.2. Percentage avoidance of the red test bead for each experimental group of chicks in (a) Experiment 1 (dose response), (b) Experiment 2 (time of injection), (c) Experiment 3 (time of test) and (d) Experiment 4 (time of reminder trial). Avoidance of the red test bead indicates memory for the training event. * = $p < 0.05$ for comparisons between 20% and 100% v/v MeA conditions for each dose (Experiment 1) and between piracetam and saline conditions for each time point (Experiment 2). As the interaction effect was non-significant in Experiments 3 and 4, comparisons between piracetam and saline were not able to be carried out.

Experiment 2: Time of injection. Hierarchical logistic regression was carried out to examine the effect of drug (saline vs. 10 mg/kg piracetam) and time of injection relative to training on avoidance of the red test bead. The main effects of drug and time of injection were entered in the first step and the drug-by-time of injection interaction term was entered in the second step. The interaction step was found to be significant ($\chi^2(7) = 17.692$, $p = 0.013$); therefore, simple main effects were investigated using Pearson's chi-square tests (drug by avoidance) at each dose of piracetam. Figure 2.2b illustrates the percentage avoidance for each experimental group. Significant drug-by-avoidance effects were found at injection times of 0 ($\chi^2(1) = 9.972$, $p = 0.002$), 5 ($\chi^2(1) = 10.252$, $p = 0.001$), 15 ($\chi^2(1) = 13.750$, $p < 0.001$), 60 ($\chi^2(1) = 4.031$, $p = 0.045$) and 120 min ($\chi^2(1) = 4.118$, $p = 0.042$) post-training, while all other comparisons were not significant ($p > 0.05$).

Experiment 3: Time of test. Hierarchical logistic regression was carried out to examine the effect of drug (saline vs. 10 mg/kg piracetam) and time of test post-training on avoidance of the red test bead. Main effects of drug and time of test were entered in the first step and the drug-by-time of test interaction term was entered in the second step. While the first step (main effects) was found to significantly improve the model ($\chi^2(8) = 33.983$, $p < 0.001$), the second step (interaction) was found to be non-significant ($\chi^2(7) = 13.130$, $p = 0.069$). The direction of the significant main effect for drug indicated greater avoidance for piracetam than saline, irrespective of the time of test. Figure 2.2c illustrates the percentage avoidance for each experimental group.

Experiment 4: Time of reminder trial. Hierarchical logistic regression was used to investigate the effect of drug (saline vs. 10 mg/kg piracetam) and time of reminder trial post-training on avoidance of the red test bead. Main effects of drug and time of reminder were entered in the first step and the drug-by-time of reminder interaction term

was entered in the second step. While the first step (main effects) was found to significantly improve the model ($\chi^2(5) = 28.087$, $p < 0.001$), the second step (interaction) was found to be non-significant ($\chi^2(4) = 5.605$, $p = 0.231$). The direction of the significant main effect for drug indicated greater avoidance for piracetam than saline, regardless of the time of reminder trial. Figure 2.2d illustrates the percentage avoidance for each experimental group.

Discussion

The results of these four experiments indicate that piracetam significantly facilitated memory in healthy day-old chicks under weak training conditions at doses between 5 and 25 mg/kg, as measured at 180 min post-training in the weak PAL task. Piracetam (10 mg/kg) facilitated memory at 180 min post-training when injected from immediately after the weak training event until 120 min after training. The facilitatory effect did not appear to be significantly mediated by the time at which testing occurred (up to 24 hours post-training). Finally, whilst administration of a reminder trial in conjunction with 10 mg/kg piracetam injected 5 min post-reminder resulted in facilitation of memory for the learning event as measured at 180 min post-reminder, the time at which the reminder trial was presented post-training did not appear to interact with this effect.

The dose-response effect of piracetam noted in Experiment 1 is generally consistent with the results of Loscertales et al. (1998), who observed memory-enhancing effects of piracetam at 10 and 50 mg/kg doses. The current study found that weak training in combination with a 5, 10 or 25 mg/kg dose of piracetam led to enhanced memory consolidation such that there was no significant difference from the strong training paradigm. However, the 50 and 100 mg/kg doses were ineffective here. The different findings at the 50 mg/kg dose between the current research and that of Loscertales et al. (1998) may be attributable to methodological differences. For example,

in Loscertales et al.'s (1998) study (and in contrast to Experiment 1 here), a 10% v/v solution of MeA was used to coat the training bead, the drug was injected 30 min post-training and retention was measured at 24 hours.

The results of the time of injection study (Experiment 2) are also largely consistent with those of Loscertales et al. (1998). In both studies, piracetam was found to be ineffective when injected prior to training, and effective when injected up to at least 60 min post-training. The current study additionally found that injection of piracetam at 120 min was effective relative to saline; however, the Loscertales et al.'s (1998) study did not. Interestingly, Mondadori, Ducret, and Borkowski (1991) found that piracetam injected up to 8 hours post-training facilitated retention in a step-down passive avoidance task in mice tested 3 days post-training. However, the endurance of a memory trace has been shown to depend on the strength of the initial learning event (Crowe, Ng, & Gibbs, 1989). It is possible that the aversant used by Mondadori et al. (1991) – a footshock – was stronger than the distastefulness of the 20% MeA solution used in the current study, which in turn was stronger than the 10% MeA solution used by Loscertales et al. (1998). Therefore, this may account for the increased training-injection interval over which piracetam may facilitate memory demonstrated by Mondadori et al. (1991), and by the present study relative to that of Loscertales et al. (1998). In another study, Mondadori and Petschke (1987) noted that 100 mg/kg piracetam, when injected 60 min prior to learning in the step-down passive avoidance task, enhanced memory in mice (relative to saline control), whereas injection immediately after learning did not. This finding is consistent with the lack of effect of such a high dose of piracetam, injected immediately after training, demonstrated by Loscertales et al. (1998) and in Experiment 1 of the present research. The effectiveness of 100 mg/kg piracetam injected 60 min prior to training may possibly be explained by partial metabolism of the drug in the time interval leading up to the training event.

The time course of piracetam's facilitation of memory in the current study (Experiment 3) is also consistent with Loscertales et al.'s (1998) findings, where a 24-hour test delay was employed and piracetam was shown to preserve avoidance in a weak version of the PAL task. Although the non-significant interaction effect in Experiment 3 precluded analysis of simple effects, it appears likely that piracetam did indeed enhance memory particularly from 40 min post-training onward. Further to this, Mondadori and Petschke (1987) also found facilitation of memory tested at 24 hours in mice using a single-trial step-down passive avoidance task, and Mondadori et al. (1991) found a facilitatory effect at 3 days post training. It appears that piracetam facilitates permanent consolidation of the memory trace.

Additionally, while the interaction between drug and time of test was found to be non-significant, the pattern of data for the saline control groups in Experiment 3 approximately supports the work of Crowe and others (e.g. Crowe & Hale, 2002, 2004; Crowe et al., 1989) in showing that weak training leads to a memory trace that lasts up to between 20 and 40 min (35-45 min according to Crowe & Hale, 2002, 2004; Crowe et al., 1989). This finding is important in confirming that under weak training conditions, testing at 3 hours post-training should not reveal intact memory unless memory has been facilitated by the intervention. However, attention must be drawn to the discrepancy of the saline group data under weak training in the current study with data from other laboratories. For example, according to Rose and colleagues, weak training on its own leads to a memory trace lasting several hours (Sandi & Rose, 1994). This difference may possibly reflect differences in procedures used in each laboratory (i.e. the size/colour of the beads used, the pre-training protocol, etc.; see Gibbs et al., 2008, for a discussion of these issues).

The combined results of Experiments 2 and 3 illuminate piracetam's effect upon memory in the context of Gibbs and Ng's three-stage sequentially-dependent model of

memory formation (Gibbs & Ng, 1976, 1977; Ng et al., 1997). To briefly summarise, the stages in Gibbs and Ng's model are: (1) a *short-term* memory (STM) stage which is developed by 5-10 min post-training and is fully decayed by about 30 min, (2) an *intermediate* or labile memory (ITM) stage which develops between 20 and 50 min post-training and decays fully after 90 min, and (3) a *long-term* memory (LTM) stage which develops at around 60 min and lasts beyond 24 hours, potentially indefinitely. Given that piracetam has been demonstrated to facilitate retention of the aversive event 3 hours after training when injected as late as 120 min after training, it can be postulated that piracetam facilitates long-term memory. At 120 min post-training, sufficient time has elapsed such that both the STM and ITM stages have occurred and decayed. At this point, the LTM stage has begun. The facilitation of memory by piracetam as late as 24 hours post-training lends further support to this postulate, since LTM is proposed to last indefinitely, whereas STM and ITM are said to last up to 30 min and 90 min, respectively (Gibbs & Ng, 1977). As to the reason why piracetam facilitates memory when injected from as early as immediately following training, it is possible that piracetam may facilitate more than one stage of memory; that is, it facilitates STM or ITM or both stages in addition to LTM.

Regarding memory reconsolidation (Experiment 4), there have been no previous studies that have directly examined piracetam's effects on the reconsolidation of memory. Nevertheless, the current finding that piracetam injected shortly after reminder enhances memory over and above a reminder trial alone is in keeping with the combined results of Przybyslawski and Sara (1997), who noted that NMDA receptor inhibitor MK-801 interfered with reconsolidation processes, and Sharma and Kulkarni (1992), who found that the memory-enhancing effect of piracetam was blocked by MK-801. That MK-801 blocks both reconsolidation and piracetam's facilitatory effect is consistent with piracetam's facilitation of reconsolidation as demonstrated in the current study, and may

potentially explain its effect here. On the other hand, Copani et al. (1992) noted that piracetam's mechanism of action involves AMPA and not NMDA receptors. Assuming an AMPA receptor-related mechanism for piracetam in reconsolidation of memory, the results of Experiment 4 would therefore be consistent with previous research by Sherry and Crowe (2008), who noted AMPA receptor involvement in the later stages of memory reconsolidation (cf. Mamou et al., 2006). The facilitation of both consolidation and reconsolidation by piracetam may be viewed as inconsistent with arguments that the two processes are different, rather than being a single process. However, it may be that piracetam's effect is at a more non-specific level that allows for consolidation and reconsolidation to be neurophysiologically distinct. Assuming the stance taken by Winblad (2005) is correct (i.e. that piracetam's mode of action is via enhancing cell membrane fluidity), piracetam may enhance both consolidation and reconsolidation, given that interfering with membrane fluidity has been shown to produce deficits in learning and memory (Clarke, Prendergast, & Terry Jr, 1999). Future research into the mechanism by which piracetam facilitates reconsolidation processes should investigate whether MK-801 (and indeed, various other inhibitory substances) impedes this particular facilitatory effect of piracetam. This would help to confirm the role of NMDA receptors in reconsolidation, and whether piracetam's role in facilitation of memory reconsolidation involves these receptors or some other mechanism entirely.

With regard to the neuroanatomical underpinnings of learning and memory in the chick, it has been demonstrated that training in the PAL task specifically is associated with activity in the intermediate medial mesopallium (e.g. Csillag, 1999). This area is also involved with other forms of learning in the chick, such as imprinting (see Horn, 1998, for a review). However, it is unclear whether reconsolidation is subserved by the same or different brain regions as original consolidation. Note that in the current study, piracetam was injected subcutaneously. Although piracetam is highly hydrophilic

(Gouliaev & Senning, 1994), the fact that the neonate chick's blood-brain barrier is yet to fully develop (Andrew, 1991) indicates that subcutaneously administered piracetam would be available to all brain regions, and as a result, those areas responsible for both consolidation and reconsolidation of memory.

Conclusions

In sum, piracetam has been demonstrated to enhance memory consolidation by delaying the decay of weak memory traces. It appears to have its effect at the long-term memory stage as defined by Gibbs and Ng's model. Piracetam was also shown to facilitate reconsolidation of previously consolidated memories made labile by the introduction of a 'reminder'. Though piracetam falls into the "AMPAkine" class of drugs, given its multiple potential modes of action, it is difficult to identify the mechanism by which piracetam facilitates memory processes in particular, and it is possible that several mechanisms may play a role in piracetam's facilitation of memory.

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Chapter 3: Memantine Facilitates Memory Consolidation and Reconsolidation in the Day-Old Chick

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Abstract

Memantine is a non-competitive *N*-methyl-D-aspartate (NMDA) receptor antagonist that has been approved for the treatment of the cognitive deficits noted in Alzheimer's disease. While there is a body of research that supports memantine's facilitative action upon memory compromise, this series of studies aimed to investigate the effects of this drug in healthy animals with intact memory functioning. A 0.1 mM dose of memantine injected immediately after a weak training event (i.e. 20% v/v methyl anthranilate) was found to enhance passive avoidance learning for this event in day-old chicks up to 24 hours following training. The same dose of memantine was also observed to enhance memory for the training event when it was administered in conjunction with a reminder trial. These results suggest that memantine is capable of facilitating both memory consolidation as well as memory reconsolidation. It was concluded that memantine's mechanism may involve the short-term or intermediate memory phases of the Gibbs and Ng model of memory, and that the current findings represent enhancement of intact memory, rather than amelioration of memory compromise.

Keywords: Memantine Passive avoidance Day-old chick Consolidation Reconsolidation

Introduction

Memantine is a non-competitive *N*-methyl-D-aspartate (NMDA) receptor antagonist currently approved for the treatment of Alzheimer's disease (AD) in the United States and Europe. In human clinical trials, memantine has been noted to improve cognition in AD as well as in vascular dementia (Orgogozo, Rigaud, Stöffler, Möbius, & Forette, 2002; Peskind et al., 2006; Winblad, Jones, Wirth, Stöffler, & Möbius, 2007; Winblad & Poritis, 1999). Memantine has also been demonstrated to enhance spatial memory in aged rats (Beracochea, Boucard, Trocme-Thibierge, & Morain, 2008). Recently, Barber and colleagues, using the day-old chick, have demonstrated that the drug can facilitate memory that has been compromised by isolation-induced stress (Barber, Meyers, & McGettigan, 2010).

While the major target of memantine's therapeutic mechanism is the NMDA receptor, memantine may also act as an antagonist at serotonin (5-HT) and nicotinic acetylcholine (ACh) receptors (see Rammes, Danysz, & Parsons, 2008; Rogawski & Wenk, 2003, for reviews). In contrast, Drever et al. (2007) have demonstrated that memantine's enhancement of synaptic transmission in the mouse hippocampus is ameliorated by application of the muscarinic ACh receptor antagonist scopolamine, and that memantine reverses the inhibitory effects of scopolamine on learning and memory. Barber and Haggarty (2010) have also shown facilitation of scopolamine-compromised memory by memantine in the day-old chick. These findings support the contention that cholinergic pathways, in addition to glutamatergic pathways, may be involved in memantine's effect on memory.

It should be noted that memantine's antagonistic effect on NMDA receptors, in addition to its facilitatory effect on memory, poses something of a paradox, as activation of NMDA receptors has been widely demonstrated to be involved in memory function (e.g. Morris, Anderson, Lynch, & Baudry, 1986; Whitlock, Heynen, Shuler, & Bear,

2006). An explanation for this seemingly contradictory mechanism has been discussed in a review by Parsons, Stöffler and Danysz (2007). These investigators have suggested that memantine's facilitation of long-term potentiation (LTP) and of memory occurs in a dose-dependent manner via restoring glutamatergic homeostasis. The authors further contend that the over-stimulation of NMDA receptors is just as detrimental to memory function as is complete or near-complete blockade, and that memantine may help to ameliorate the inhibitory effect caused by over-stimulation.

The effect of memantine on unimpaired subjects has also been investigated; however, the findings have been less conclusive than those noted in studies of memory-compromised subjects. A recent review has suggested that memantine either has no effect or results in impairment of memory in healthy subjects (Repantis, Laisney, & Heuser, 2010). Similarly, in some animal studies, various doses of memantine have been shown to either have no effect or to impair memory in rats (Réus et al., 2008) and day-old chicks (Barber et al., 2010). In contrast, other research using healthy rats has noted a facilitatory effect of memantine on memory functioning (Wise & Lichtman, 2007; Zoladz et al., 2006) and improved maintenance of hippocampal LTP *in vivo* (C. A. Barnes, Danysz, & Parsons, 1996). The effect of memantine on intact memory thus continues to be a topic of contention.

Another issue of relevance in the contemporary study of memory is the effect of memantine on the process of reconsolidation. Reconsolidation refers to the process that takes place once a previously-consolidated memory is retrieved from storage and once again becomes transformed into a labile and modifiable state. The reactivated trace is then consolidated once more, or *reconsolidated* in a state that includes the additional information acquired at the time of reactivation (for a review, see Tronson & Taylor, 2007). To date, only few studies have investigated memantine's effect on the process of reconsolidation, and in general, this evidence has indicated an inhibitory effect. Popik,

Wrobel and Bisaga (2006) found that memantine, when injected at the time of a retrieval phase (i.e. at the time when the memory is recalled and made labile), prevented morphine-primed reinstatement of morphine-conditioned place preference in rats. This suggests that memantine interfered with reconsolidation such that the memory of place preference in the rats was not able to be recalled later at the time of testing, even when primed by the injection of morphine.

The aim of this series of studies was to investigate memantine's function in healthy day-old chicks, using both a weak and a strong aversive training experience and employing both consolidation and reconsolidation trials, in the context of a well studied training paradigm. The weak passive avoidance learning (PAL) task was employed in combination with the day-old chick, a paradigm that has been extensively studied (e.g. Crowe & Hale, 2002). To date, no published study has examined the effect of memantine on the weak PAL task. The specific aims of the research were as follows: to determine the most effective dose of memantine necessary to enhance intact memory using a weak training experience (Experiment 1); to determine the time window over which injection of memantine was effective (Experiment 2); to investigate the length of time over which memantine had its facilitatory effect (Experiment 3), and; to investigate the ability of memantine to enhance memory reconsolidation (Experiment 4).

Method

Subjects. One-thousand one-hundred and sixty male day-old New Hampshire × White Leghorn chicks (*Gallus domesticus*; average weight of 45g) were employed as subjects in the experiments: 240 each in Experiments 1 and 4, 360 in Experiment 2, and 320 in Experiment 3. The chicks were housed in wooden boxes (20 × 25 × 20cm) with open tops and were kept in pairs so as to reduce any distress brought about by social isolation. The chicks were kept warm by a 60W incandescent light bulb positioned above each box, and chick mash was provided *ad libitum*. For the purpose of identification

during data collection, one chick in each pair was marked on the head with a black felt tip pen. Chicks were left alone to settle for at least 30 min prior to experimentation. Each experimental condition initially consisted of a group of 20 chicks, but depending on the number of chicks that successfully completed the baseline and training phases, the final number varied. Approximately 20% of chicks were excluded from analysis on the basis of failure to peck at the bead during the baseline and training phases.

Materials. Memantine (3,5-dimethyladamantan-1-amine) hydrochloride was injected subcutaneously in a volume of 0.1 mL per chick in all four experiments, using a 1 mL syringe with a 27 gauge needle. In Experiment 1, doses used were 0.1, 0.5, 1, 2 and 3 mM of memantine, and in Experiments 2–4, a dose of 0.1 mM was employed. Saline solution was injected in the control conditions. The PAL task required a chrome bead and two red glass beads (each fixed to the end of a wire rod) and the aversive substance methyl anthranilate (MeA). Where MeA was used at a concentration of 20% v/v, the MeA was diluted in ethanol. The number of pecks for each chick in each condition was recorded using a handset connected to a PC in the laboratory so that button presses on the handset could be recorded and summed automatically.

Procedure and design. All four experiments employed the PAL task, first introduced by Cherkin (1969), that exploits the chick's natural inclination to peck at novel objects. The aim of the task is to train chicks to avoid pecking at a target bead, and it is intended to measure intact memory. The phases of the task are as follows: pretraining, in which chicks are presented twice with a water-coated chrome bead to encourage a pecking response; baseline, in which a water-coated red bead is presented and pecks recorded as a baseline measure; training, in which a second red bead coated with the aversive substance methyl anthranilate (MeA) is presented to the chicks; reminder (Experiment 4 only), in which chicks are shown a dry red bead but are not allowed to peck, in order to elicit recall for the training event; and test, in which chicks

are exposed to a dry red bead as a measure of retention of the training. Refer to Figure 3.1 for an outline of the timing of the phases in each of the four experiments, and see Crowe and Hale (2004) for further details on the PAL procedure. All phases involved a 10-s exposure to the bead only. Chicks that did not peck at either the baseline or training phases were excluded from the subsequent analysis.

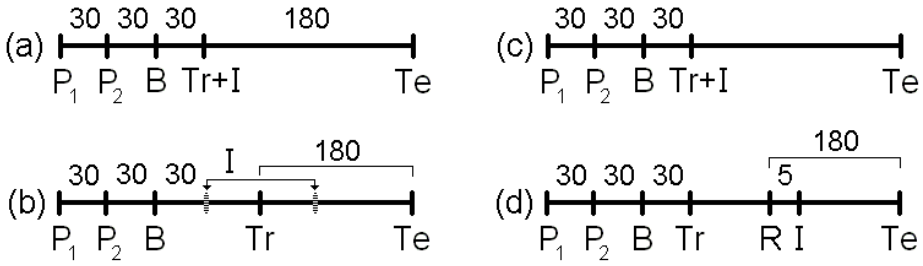

Figure 3.1. Timeline of events for (a) Experiment 1 (dose response), (b) Experiment 2 (time of injection), (c) Experiment 3 (time of test) and (d) Experiment 4 (time of reminder trial). Numbers above the lines indicate minutes between events marked on the lines. P = pretraining, B = baseline, Tr = training, I = injection, R = reminder, Te = test.

All independent variables (IVs) were categorical in nature. In Experiment 1, the IVs were concentration of MeA at training and dose of memantine. In Experiment 2, the IVs were time of injection relative to the training phase and drug injected (saline vs. memantine). In Experiment 3, the IVs were time of test after the training phase and drug injected. In Experiment 4, the IVs were time of reminder trial after the training phase and injected drug. In all experiments, the dependent variable was a binary measure with the two levels coded as avoidance (i.e. no pecks to the red test bead, demonstrating memory for the training event) or no avoidance (i.e. one or more pecks to the red test bead, demonstrating a lack of memory for the training event). While previous studies using the

PAL task have employed a number of dependent variables (see Crowe & Hamalainen, 2001; Gibbs, Johnston, Mileusnic, & Crowe, 2008), the binary measure described here was employed due to significant skew of the originally calculated continuous measure (an avoidance ratio; e.g. Crowe & Hale, 2004).

Results

Experiment 1: Dose-response. Hierarchical logistic regression was used to examine the impact of dose of memantine and concentration of MeA (20% vs. 100% v/v) on avoidance at test. Main effects of dose and MeA were entered in the first step, and in the second step, the dose-by-MeA interaction term was entered. The interaction step was significant ($\chi^2(5) = 14.421$, $p = 0.013$) hence simple main effects were investigated using Pearson chi-square tests (MeA-by-avoidance) at each dose of memantine. Figure 3.2a presents the percentage of chicks in each experimental group that avoided the red test bead (and thus demonstrated intact memory). The single, non-significant MeA-by-avoidance cross-tabulation for 0.1 mM memantine ($p > 0.05$) indicates effective facilitation of memory at this dose – that is, injection of 0.1 mM memantine rendered the normally 'weak' training paradigm of 20% MeA equivalent to the 'strong' 100% MeA training paradigm. Thus, the 0.1 mM dose of memantine was subsequently employed in Experiments 2–4.

Experiment 2: Time of injection. Hierarchical logistic regression was employed to examine the effect of time of injection of drug relative to training and drug injected (saline vs. 0.1 mM memantine) on avoidance. Again, main effects were entered in the first step and the interaction term was entered in the second step. The main effects in the first step were found to significantly improve the model ($\chi^2(9) = 56.044$, $p < 0.001$); however, the interaction step was found to be non-significant ($p > 0.05$). Therefore, simple main effects at each time of injection were not able to be investigated. The

direction of the significant main effect for drug indicated greater avoidance for memantine than saline. Figure 3.2b presents the percentage avoidance for each group.

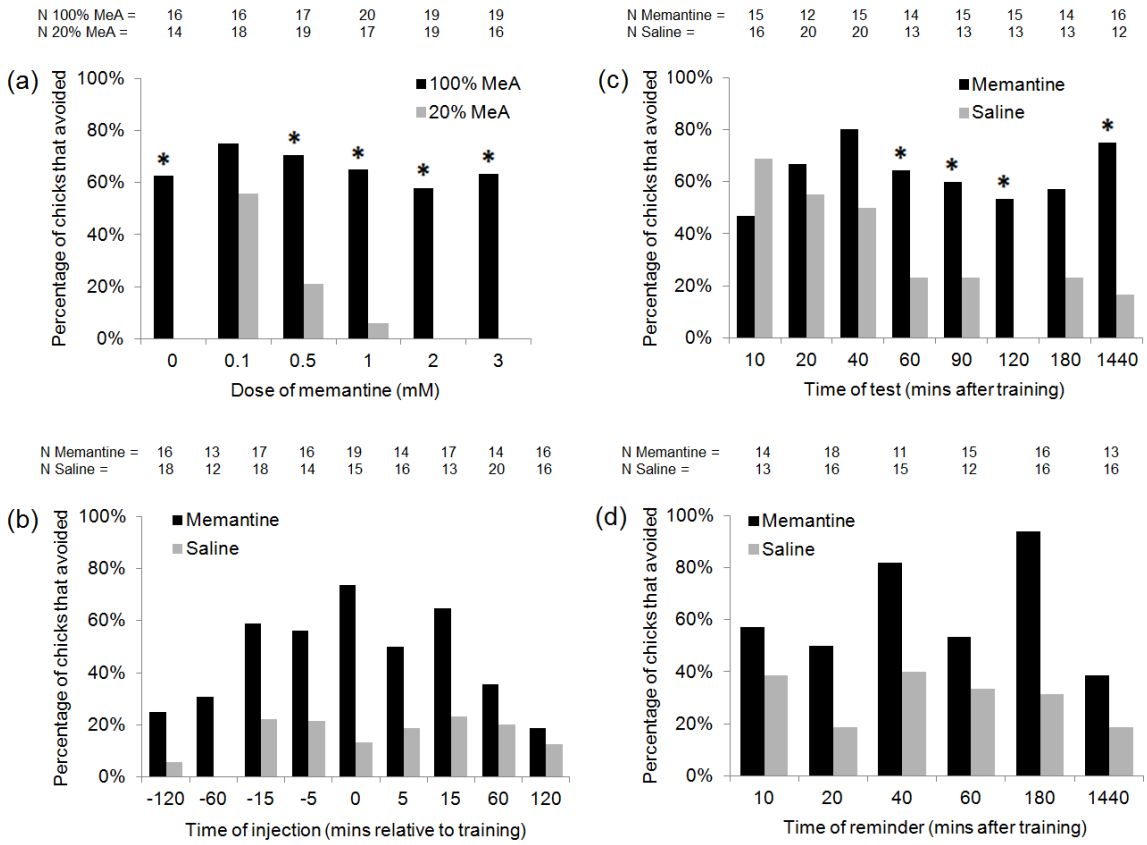

Figure 3.2. Percentage avoidance of the red test bead for each group of chicks in (a) Experiment 1 (dose response), (b) Experiment 2 (time of injection), (c) Experiment 3 (time of test) and (d) Experiment 4 (time of reminder trial). Avoidance indicates memory for the training event. * = $p < 0.05$ for comparisons between 20% and 100% v/v MeA conditions for each dose (Experiment 1) and between saline and memantine conditions for each time point (Experiment 3). As the interaction effect was non-significant in Experiments 2 and 4, comparisons between memantine and saline were not able to be carried out.

Experiment 3: Time of test. Hierarchical logistic regression was used to investigate the effect of time of test after training and drug injected (saline vs. 0.1 mM memantine) on avoidance. Again, main effects were entered in the first step and the

interaction term was entered in the second step. The interaction step was found to be significant ($\chi^2(7) = 18.625$, $p = 0.009$); thus, simple main effects were investigated using Pearson chi-square tests (drug-by-avoidance) at each time of test. Significant drug-by-avoidance cross-tabulations were found at test times of 60 min ($\chi^2(1) = 4.636$, $p = 0.031$), 90 min ($\chi^2(1) = 3.877$, $p = 0.049$), 120 min ($\chi^2(1) = 9.707$, $p = 0.002$) and 1440 min ($\chi^2(1) = 9.333$, $p = 0.002$) post-training. Figure 3.2c illustrates the percentage avoidance for each group.

Experiment 4: Time of reminder trial. Hierarchical logistic regression was carried out to examine the effect of time of reminder trial post-training and drug injected (saline vs. 0.1 mM memantine) on avoidance. Once again, main effects were entered as the first step and the interaction term was entered as the second step. The interaction step was found to be non-significant ($p > 0.05$), precluding analysis of simple main effects at each time of reminder trial; however, the main effects step was significant ($\chi^2(6) = 31.553$, $p < 0.001$). The direction of the significant main effect for drug indicated greater avoidance for memantine than saline. Figure 3.2d illustrates the percentage avoidance for each experimental group.

Discussion

The findings of these experiments indicate that memantine is most effective in facilitating memory for the PAL task in the day-old chick at a dose of 0.1 mM, and that this facilitation is not significantly moderated by the time at which the injection is administered. This dose effectively enhances memory when measured at least up to 24 hours post-training. Additionally, 0.1 mM memantine also facilitated recall for a weak training event when administered in conjunction with a reminder trial, which indicates that memantine is also capable of facilitating the process of reconsolidation.

To date there has been no study that examines memantine's effect on the weak version of the PAL task. Barber et al. (2010) used the strong version of this task (i.e. with 100% MeA v/v) and found that memantine inhibited memory at dose of 15 mM, while having no effect at lower doses. The findings of Experiment 1 are consistent with Barber et al.'s (2010) results in that memory for the strong training event was observed to be unaffected following memantine treatment at doses of up to 3 mM. It is likely that use of the strong training paradigm led to a ceiling effect in both Barber et al.'s (2010) study and the current research, given that a high proportion of saline-treated chicks avoided the test bead in both cases. In contrast, no saline-treated chicks avoided the test bead in the current study when the weak training paradigm was employed. This may be explained by the observation that increasing the strength of the training event leads to better endurance of a memory trace (Crowe, Ng, & Gibbs, 1989). Thus, the strong training condition may not be the optimal training intensity with which to investigate a possible facilitatory effect of memantine. Moreover, the lowest dose of memantine used by Barber et al. (2010) in their non-stressed experiment was 0.5 mM, whereas the current study employed a dose of 0.1 mM – the only dose at which memantine was found to be effective in facilitating memory. This could also explain the lack of facilitation noted in Barber et al.'s (2010) research.

To account for the current finding of memory facilitation, one could consider the theory put forward by Parsons et al. (2007) that memantine facilitates memory by ameliorating excess (i.e. pathological) glutamatergic stimulation. For example, Zoladz et al. (2006) found a facilitatory effect of memantine in healthy adult rats trained on a radial-arm water maze task. They postulate that memantine's effect under these circumstances may be explained by the fact that the task involved a component of stress to the rats (i.e. immersion in cold water). Imposing stress on animals has been demonstrated to impair memory performance (e.g. Park, Zoladz, Conrad, Fleshner, &

Diamond, 2008), and so, rather than enhancing memory, memantine may have actually rescued the memory impaired by stress in Zoladz et al.'s (2006) rats. This explanation is in keeping with the large body of research demonstrating enhancement of impaired memory by memantine noted earlier. Thus, one explanation for the present findings may be that some component of the PAL task imposed stress upon the chicks (e.g. the injection procedure, exposure to the aversive substance MeA) leading to baseline impairment of memory ability that was rescued (rather than enhanced) by memantine.

However, previous data from our laboratory and others invalidates this explanation. It has been demonstrated that memory for the weak PAL task in chicks does not differ markedly between saline-injected and non-injected control groups (Crowe, Ng, & Gibbs, 1990; Johnston, Clements, & Rose, 1999). Furthermore, stress and stress-related factors in the weak PAL task have actually been shown to facilitate memory (Crowe et al., 1990; Johnston & Rose, 1998; Sandi & Rose, 1994). Since it is unlikely that the testing procedures used here would be as stressful as other experimental manipulations shown to enhance memory (e.g. an hour of social isolation), there is certainly cause to doubt that the testing procedures caused such a degree of stress as to impair memory. Therefore, it seems most likely that memantine actually enhanced intact memory in these chicks.

The facilitation of memory in healthy chicks by memantine, as found in Experiments 1–3, corroborates the findings of Wise and Lichtman (2007). These investigators reported that a low dose of memantine (0.3 or 0.56 mg/kg) significantly reduced errors on a delayed radial-arm maze task when administered to healthy adult rats prior to training. It should be noted that the even lower dose (0.1 mg/kg, still greater than the dose used here – 0.1 mM, approximately equivalent to 0.048 mg/kg in the chick) failed to enhance memory in Wise and Lichtman's (2007) study, although this discrepancy with the current study may reflect species-related differences.

Given that memantine's facilitation of memory in the present research does not appear to involve blockade of excess glutamatergic stimulation at NMDA receptors, as discussed above, the possibility of a role for another receptor type must be considered. For example, Drever et al. (2007) noted an enhancing effect of memantine upon synaptic transmission that was ameliorated by the muscarinic ACh receptor antagonist scopolamine but not the NMDA receptor antagonist MK-801. This is suggestive of muscarinic receptor activation by memantine. Rammes, Rupprecht, Ferrari, Zieglgänsberger and Parsons (2001) have demonstrated an antagonistic action of memantine on 5-HT_3 receptors expressed in cell culture; as antagonism of these receptors has been shown to improve memory and learning (J. M. Barnes et al., 1990; Chugh, Saha, Sankaranarayanan, & Sharma, 1991; Pitsikas, Brambilla, & Borsini, 1994), this could represent an alternative possible mechanism for memantine.

The results of Experiment 3 also serve to corroborate previous saline control data in the PAL task in the day-old chick from our laboratory. The current results indicate that memantine facilitated memory retention up to at least 24 hours post-training, whereas chicks in the control condition experienced decay of the memory trace beyond around 40 min after training. This finding is comparable to those of Crowe and colleagues (e.g. Crowe & Hale, 2002, 2004; Crowe et al., 1989). It should be noted that other laboratories have found memory retention lasting several hours post-training in the weak PAL task (e.g. Sandi & Rose, 1994), although differences in findings between laboratories have been attributed to variation in experimental protocol (see Gibbs et al., 2008 for a review).

Although no significant interaction was found in Experiment 2 (time of injection by drug injected), the fact that memantine was shown to significantly enhance memory when injected immediately after training (as in Experiments 1 and 3) suggests that memantine may act at the short-term memory phase of the Gibbs and Ng three-phase model of memory (Gibbs & Ng, 1976, 1977; Ng et al., 1997). This model has been

developed experimentally using the PAL task in the day-old chick. Briefly, the three phases of the model are: (1) short-term memory (STM), lasting from the time of training until about 10 min later, (2) intermediate memory (ITM), lasting from between 20 and 50 min post-training until about 60 min, and (3) long-term memory (LTM), lasting from around 60 min after training up until beyond 24 hours (potentially indefinitely). It is also possible that memantine may influence the ITM phase of the model, given the brevity of the STM phase and assuming that memantine injected immediately after training has not completely metabolised by 20 min post-training. However, the results of Experiment 2 do not allow the determination of whether STM or ITM processes (or a combination of both) are involved in memantine's action.

The results of Experiment 4 (time of reminder trial) indicate that memantine enhanced memory reconsolidation overall, regardless of the latency of reminder trial presentation. Previous research investigating the effect of memantine on reconsolidation has revealed an inhibitory effect (Popik et al., 2006), and the present finding is in opposition to this. Popik and colleagues, however, injected rats with a dose of memantine that, in addition to suppressing reconsolidation, failed to enhance learning in a separate memory task. The dose of memantine used in Popik et al.'s (2006) study (7.5 mg/kg) was substantially larger than the one used here (around 0.048 mg/kg), so potentially, memantine was administered at a dose too high to reveal facilitation in the former case. This is consistent with the failure of memantine to enhance weakly reinforced learning at higher doses, as noted in Experiment 1. Thus, despite Popik et al.'s (2006) findings, the dose employed in these studies may allow for facilitation of reconsolidation by memantine.

It is suggested here that memantine may have acted via a mechanism not related to NMDA receptors to enhance intact memory in the current study. It could also, therefore, have facilitated reconsolidation through such an alternative mechanism. For

example, there is evidence linking cholinergic neurotransmission with reconsolidation processes. Boccia and colleagues found that inhibition of ACh synthesis led to deficits in reconsolidation of an inhibitory avoidance memory in mice (Boccia, Acosta, Blake, & Baratti, 2004). This is in line with the suggestion that memantine may positively modulate ACh receptors, as described above. There is also some indication that 5-HT_3 receptors may be involved in reconsolidation. Receptor blockade by the antagonist ondansetron, administered in combination with the dopaminergic agonist pergolide, leads to inhibition of the reconsolidation of methamphetamine sensitisation in rats experiencing withdrawal (Bhatia, Szabo, Fowler, Wetsel, & Lee, 2011). Therefore, the memantine-induced enhancement of reconsolidation in the current study is not consistent with a 5-HT_3 receptor-mediated mechanism. Whether memantine facilitates reconsolidation by the same mechanism by which it facilitates consolidation remains to be determined, although this would be consistent with the notion of reconsolidation as a partial recapitulation of the consolidation processes (e.g. Alberini, 2005).

Conclusions

The current research has demonstrated that memantine enhances memory consolidation and reconsolidation when administered at a relatively low dose. The drug appears to act during either the STM or ITM phases of the Gibbs and Ng model of memory, or potentially both. It is argued that memantine's mode of action involves enhancing normal memory, given that the subjects in the current study were young, healthy, non-stressed animals. Further investigation is required to elucidate the biochemical mechanisms by which memantine facilitates consolidation and reconsolidation. It is clear, however, that memantine's various effects are crucially dependent upon dosage.

Acknowledgements

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Chapter 4: Brain-Derived Neurotrophic Factor Facilitates Memory Consolidation and Reconsolidation of a Weak Training Stimulus in the Day-Old Chick

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Abstract

Recent research has pointed to a role for brain-derived neurotrophic factor (BDNF) in long-term potentiation and memory. The present series of experiments examined the effects of the application of exogenous BDNF on memory consolidation and reconsolidation of a weak training stimulus with the day-old chick, using the passive avoidance learning paradigm. Chicks injected intracranially with 12.5 μg/mL recombinant BDNF immediately after a single-trial training event displayed enhanced retention relative to saline up to 24 hours post-training. Furthermore, this dose was also shown to enhance retention when injected after a reminder trial, the purpose of which was to induce recall. Thus, exogenous BDNF was shown to enhance both consolidation and reconsolidation of memory when administered acutely to the day-old chick.

Keywords: Brain-derived neurotrophic factor Passive avoidance Day-old chick Consolidation Reconsolidation

Introduction

Brain-derived neurotrophic factor (BDNF) is a member of the class of endogenous neurotrophins, which are responsible for fostering neuronal survival and differentiation. The last two decades have seen the emergence of a body of evidence that suggests that BDNF also plays a role in activity-dependent synaptic plasticity (for reviews, see McAllister, Katz, & Lo, 1999; Poo, 2001), such as long-term potentiation (LTP). Given the widely acknowledged role of LTP in learning and memory (e.g. Whitlock, Heynen, Shuler, & Bear, 2006), it is not surprising that BDNF has also been concluded to play an important role in these events (Tyler, Alonso, Bramham, & Pozzo-Miller, 2002; Yamada, Mizuno, & Nabeshima, 2002).

At the cellular level, BDNF has been shown to enhance LTP induced by tetanic stimulation in the rat hippocampus *in vitro* (Figurov, Pozzo-Miller, Olafsson, Wang, & Lu, 1996). Induction of LTP in rat hippocampal slices has been demonstrated to result in a significant increase in BDNF mRNA levels (Patterson, Grover, Schwartzkroin, & Bothwell, 1992). It has also been demonstrated that the hippocampal slices of BDNF-deficient mice display markedly impaired LTP (Korte et al., 1995), and that the application of recombinant BDNF reverses this deficit (Patterson et al., 1996), suggesting a role for BDNF in neuronal plasticity at the acute level.

At the behavioural level, several studies have indicated that BDNF is implicated in memory processing; injection of BDNF antibodies (Alonso et al., 2002; Johnston, Clements, & Rose, 1999) and deletion of the BDNF gene in mouse hippocampi (Heldt, Stanek, Chhatwal, & Ressler, 2007) have been shown to lead to impaired memory retention in experimental animals. Johnston et al. (1999) also found that day-old chicks injected intracranially with recombinant BDNF displayed better memory for a passive avoidance learning (PAL) event relative to a saline-injected control group, and Alonso et al. (2002) noted an increase in BDNF mRNA expression one hour following foot-shock

training in rats. It has been demonstrated that administration of BDNF antisense oligodeoxynucleotides (ODN) leads to impairments in long-term memory retention (Johnston & Rose, 2001; Lee, Everitt, & Thomas, 2004). In contrast, Cirulli et al. (2000) have noted that rats trained over three days on a water maze task and injected with BDNF displayed no better retention two days later than did vehicle-injected controls or animals injected with BDNF antibodies. Bekinschtein and colleagues have also recently demonstrated a role for BDNF in the persistence of long-term memory storage (Bekinschtein et al., 2007; Bekinschtein et al., 2008). Taken together, these findings indicate reasonably consistent support for a role for BDNF in both acute and longer-term synaptic modification.

Another important issue in contemporary memory research is that of memory reconsolidation. This term refers to the process occurring after information has been retrieved from long-term storage and transformed into a labile state. Retrieval of a memory trace from storage may be brought about by the administration of a reminder trial. At this point, the retrieved memory trace is able to be modified and then reconsolidated (for review, see Tronson & Taylor, 2007). The role of BDNF in memory reconsolidation has been investigated previously. Lee et al. (2004) employed a contextual fear paradigm and noted that while BDNF is required for consolidation, it does not appear to be required for reconsolidation. However, only limited research has examined BDNF with respect to reconsolidation thus far, and so further study is indicated.

The current study set out to investigate the effects of the application of exogenous BDNF on memory in the day-old chick, with particular interest in effects following acute administration. The “weak” version of the PAL task (i.e. using a weak aversant at training) was employed here. The aims were threefold: (1) to determine an effective dose range in which injected BDNF may be expected to facilitate memory; (2) to determine the time period following the learning event over which BDNF successfully enhances

memory; (3) to determine if injected BDNF leads to facilitation of memory reconsolidation. Results are discussed in light of the Gibbs and Ng three-phase model of memory in the day-old chick (Gibbs & Ng, 1976, 1977; Ng et al., 1997).

Method

Subjects. Two hundred and forty male day-old New Hampshire × White Leghorn chicks (*Gallus domesticus*; average weight of 45 g) served as subjects: four groups of 10 in Experiment 1 and 10 groups of 10 in each of Experiments 2 and 3. Chicks were kept and tested in pairs to reduce stress brought about by social isolation. Refer to Crowe and Hale (2002) for further details on animal housing. All experiments used a fully between-subjects design; each chick participated in a single experimental condition. While each experimental group initially consisted of a group of 10 chicks, those that did not successfully complete the baseline and training phases were excluded from the final analysis. The small group size was used due to financial constraints.

Materials. In all three experiments, recombinant BDNF (Abcam P/L) was injected intracranially into both hemispheres of each chick in a volume of 10 μL per hemisphere (i.e. 20 μL per chick), using a 500 μL Hamilton repeated dispensing syringe with a 27 gauge needle. In Experiment 1, doses of BDNF used were 0, 6.25, 12.5 and 25 μg/mL, and the dose of 12.5 μg/mL was used in Experiments 2 and 3. Injections were targeted at the intermediate medial mesopallium, an area previously demonstrated to be involved in memory formation in the PAL task in chicks (Csillag, 1999). Bony landmarks on the skull were used to guide freehand injection. The upper portion of the needle was covered with tubing to regulate the depth of injection to 3.5 mm. Saline solution was injected as the control substance. Materials required for administration of the PAL task are described in Crowe and Hale (2004).

Procedure and design. All experiments made use of the PAL task (Cherkin, 1969; Crowe & Hale, 2002), the object of which is to train chicks to avoid pecking at a

red bead. Briefly, the phases of the task are: pretraining (a water-coated chrome bead is presented to promote pecking in general); baseline (chicks are presented with a water-coated red bead and pecks to the bead recorded); training (chicks are exposed to a second red bead, coated with the aversive substance methyl anthranilate [MeA] diluted with ethanol to 20% v/v, and pecks are recorded); reminder (Experiment 3 only; chicks are presented with a dry red bead but are not allowed to peck it with a view to eliciting recall for the training event); test (chicks are exposed to a dry red bead as a measure of recall of the training event, and pecks are recorded). See Crowe and Hale (2004) for a more detailed description of the PAL task procedure. Figure 4.1 illustrates the timeline of phases in the PAL task for each experiment.

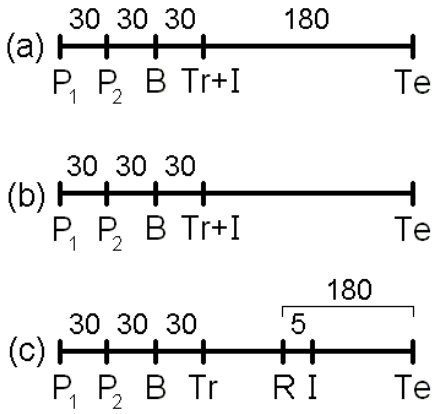

Figure 4.1. Timeline of events for (a) Experiment 1, (b) Experiment 2 and (c) Experiment 3. Numbers above the lines indicate minutes between events marked on the lines. P = pretraining; B = baseline; Tr = training; I = injection; R = reminder; Te = test.

The independent variables, which were all categorical in nature, are as follows: in Experiment 1, dose of BDNF; in Experiment 2, time of test after the training phase and drug injected (saline vs. BDNF); in Experiment 3, time of reminder trial after the training

phase and drug injected (saline vs. BDNF). In all three experiments, the dependent measure was binary. The two levels were coded as avoidance (no pecks to the red test bead, indicating memory for the training event) or no avoidance (one or more pecks to the red test bead, indicating no memory for the training event). Previous research using the PAL task has employed a range of dependent measures (i.e. percentage avoidance, discrimination ratio, avoidance ratio; Crowe & Hamalainen, 2001; Gibbs, Johnston, Mileusnic, & Crowe, 2008); however, the binary measure was employed in the current study due to substantial skew of the previously calculated continuous dependent variable (originally, an avoidance ratio). Results are presented here as percentage avoidance, calculated as the proportion of chicks in each group that did not peck at the red test bead.

Results

Experiment 1: Dose-response. Logistic regression was employed to investigate the effect of dose of BDNF on avoidance of the test bead. The model was significant ($\chi^2(3) = 10.424$, $p = 0.015$), and as a result, Pearson chi-square tests were used to compare saline with each of the three doses of BDNF. Figure 4.2a illustrates the percentage of chicks in each experimental group that avoided the red test bead (and thus demonstrated intact memory). All three doses of BDNF significantly enhanced memory relative to saline (6.25 μg/mL: $\chi^2(1) = 6.563$, $p = 0.010$; 12.5 μg/mL: $\chi^2(1) = 4.773$, $p = 0.029$; 25 μg/mL: $\chi^2(1) = 6.563$, $p = 0.010$). The 12.5 μg/mL dose, being the median of the effective doses, was therefore employed in Experiments 2 and 3.

Experiment 2: Time of test. Hierarchical logistic regression was carried out to examine the effects of time of test after training and drug injected (saline vs. 12.5 μg/mL BDNF) on avoidance of the test bead. Main effects were entered in the first step and the interaction term was entered in the second step. The interaction step was found to be significant ($\chi^2(4) = 10.447$, $p = 0.034$); thus, simple main effects were investigated using

Pearson chi-square tests (drug-by-avoidance) at each time of test. Significant drug-by-avoidance cross-tabulations were found at test times of 180 min ($\chi^2(1) = 9.899$, $p = 0.002$) and 1440 min ($\chi^2(1) = 6.537$, $p = 0.011$) post-training. Drug-by-avoidance cross-tabulations were non-significant at all other time points ($p > 0.05$). It should be

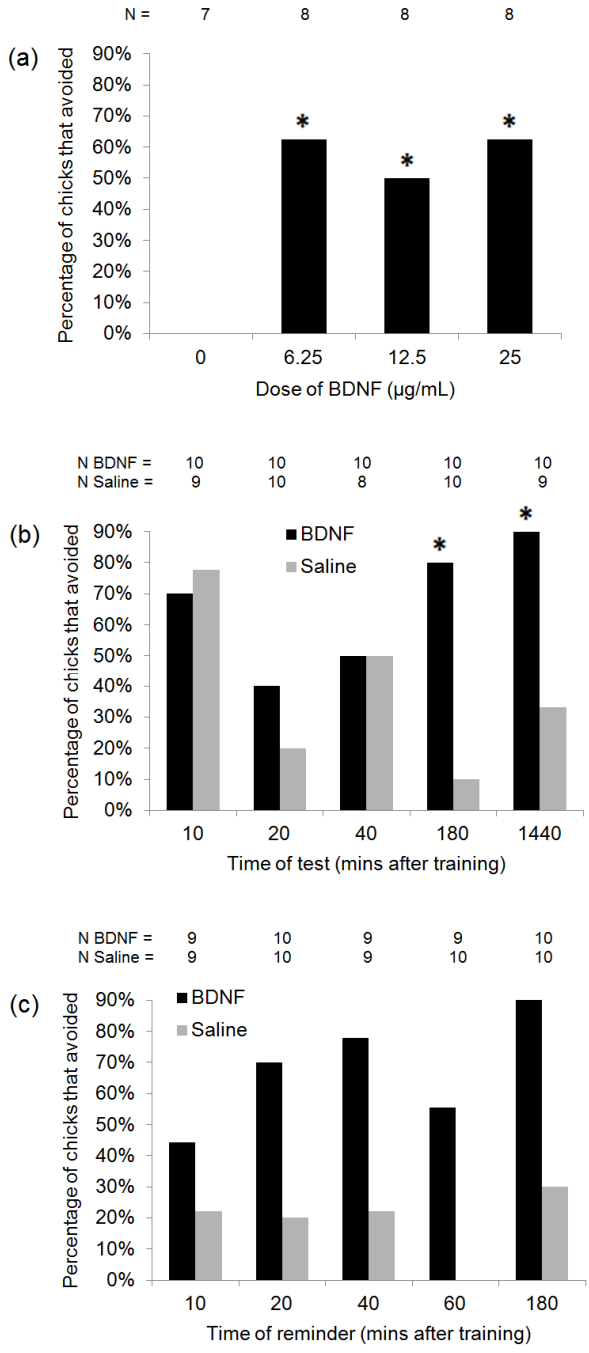

Figure 4.2. Percentage avoidance of the red test bead for each group of chicks in (a) Experiment 1, (b) Experiment 2 and (c) Experiment 3. Avoidance indicates memory for the training event. * = $p < 0.05$.

noted that although the levels of avoidance at 10, 20 and 40 min post training were not significantly different from each other, they were somewhat lower than our previously observed levels (Crowe & Hale, 2004; Crowe, Ng, & Gibbs, 1989, 1990), probably due to the smaller sample sizes employed in this study. Figure 4.2b illustrates the percentage avoidance for each group.

Experiment 3: Time of reminder trial. Hierarchical logistic regression was used to examine the impact of time of reminder trial post-training and drug injected (saline vs. 12.5 μg/mL BDNF) on avoidance. Again, main effects were entered in the first step and the interaction term was entered in the second step. The interaction step was found to be non-significant ($\chi^2(4) = 3.679$, $p = 0.451$), although the main effects step was significant ($\chi^2(5) = 31.956$, $p < 0.001$). Thus, the main effect of the drug was significant, but this effect was not significantly mediated by the time of reminder trial. Figure 4.2c illustrates the percentage avoidance for each experimental group.

Discussion

The findings of this series of studies indicate that BDNF injected immediately after training is effective in enhancing memory for the PAL task in the day-old chick at doses ranging between 6.25 and 25 μg/mL. The 12.5 μg/mL dose effectively facilitates memory when measured up to 24 hours after the training event. Moreover, 12.5 μg/mL BDNF enhances memory recall relative to saline when injected in combination with a reminder trial, which indicates that BDNF may be involved in the process of memory reconsolidation.

These data are generally consistent with those of Johnston et al. (1999), who also examined passive avoidance learning in the day-old chick following injection of recombinant BDNF. They found that injection of a dose of 0.5 μg BDNF per chick prior to weak training (using 10% v/v MeA) led to greater avoidance than various control conditions 24 hours later. Similarly, the present research demonstrated effective

enhancement of avoidance at the same dose 3 hours post-training (Experiment 1), as well as with a smaller dose (12.5 μg/mL = 0.25 μg BDNF per chick) at 24 hours post-training (Experiment 2), with injection occurring immediately after training. Thus, the current research serves to confirm the facilitatory effect of BDNF upon memory in the day-old chick as previously reported by Johnston et al. (1999).

Experiments 1 and 2 are in partial accord with studies from the rodent literature involving injection of BDNF (Alonso et al., 2002). These authors used an inhibitory avoidance paradigm in which rats were trained to avoid stepping down onto a platform through which a foot shock was delivered. They found that 0.5 μg BDNF per rat (between 2.0 and 3.1 μg/kg) injected either 15 min before or immediately after training led to greater latency to step down (i.e. enhanced memory retention) at 1.5 hours post-training. This is congruent with current findings in that injection of BDNF (12.5 μg/mL, or 5.6 μg/kg) immediately after training led to enhancement of avoidance learning in chicks tested at a similar time (i.e. 3 hours post-training). However, in contrast to the current studies, Alonso et al. (2002) found that when BDNF was injected 15 min before or immediately after training and testing took place at 24 hours post-training, there was no enhancement of memory relative to saline at test. They did, however, find facilitation at 24 hours when injection occurred at 1 or 4 hours post-training. So, while the current finding of memory enhancement measured at 3 hours after training may be considered comparable to the findings of Alonso et al. (2002), the facilitation found here at 24 hours post-training is inconsistent with Alonso et al.'s (2002) results.

Furthermore, the data of the current research contrast with other studies that have failed to find improved memory functioning following injection of recombinant BDNF. Cirulli et al. (2000) found that rats treated with BDNF performed no better on a water maze task than did saline-treated control animals. However, there are two points of difference with the current research to consider here. Firstly, Cirulli et al.'s (2000) rats

were trained on the water maze task over either two or three days and injected at the end of training. Thus, by the time of injection, learning may have already occurred in these rats such that BDNF was administered too late in the process of memory formation (e.g. 2-3 days post-training) for facilitation to be possible. Secondly, the route of administration of BDNF was intracerebroventricular. It is possible that the substance did not adequately reach the rat hippocampi and thus was not able to exert its facilitatory effect. This second issue was in fact discussed and addressed by Cirulli and colleagues, who later performed further studies in the rat and utilised an intrahippocampal route of administration of BDNF (Cirulli, Berry, Chiarotti, & Alleva, 2004). These rats were again trained on a water maze task. Treatment with BDNF was shown to improve performance on reversal learning, but it did not improve retention on a probe trial following initial acquisition of the task. Note again that injection of BDNF occurred after the six acquisition trials spaced 20 min apart (i.e. around 100 min after training began), whereas the reversal phase occurred subsequent to BDNF administration. Thus, differences in time of injection relative to the training event may account for the differences between the present research and the work of Cirulli and others (Cirulli et al., 2000; Cirulli et al., 2004).

A follow-on issue from this discussion is the role of BDNF in the various stages of memory formation, with reference to the Gibbs and Ng three-phase model of memory formation in the day-old chick (Gibbs & Ng, 1976, 1977; Ng et al., 1997). Briefly, the three phases of the model are short-term memory (STM; 0-10 min post-training), intermediate memory (ITM; 20-50 min post-training, separable into sub-phases A and B), and long-term memory (LTM; 60 min post-training onward). The present research together with the findings of Johnston et al. (1999) indicate that BDNF plays a role in STM in the day-old chick, given that administration of BDNF occurred either prior to or immediately following the learning event. On the other hand, the persistence of the

memory enhancement seen here may be taken as evidence that BDNF plays a role in facilitating LTM. With a weak training stimulus (Crowe et al., 1990), saline injection only leads to memory that corresponds to the STM and ITM(A) phases, whereas administration of BDNF led to memory that lasted beyond this time, corresponding to the establishment of the ITM(B) and LTM phases. While the current study did not directly address the biochemical mechanism of this process, it has previously been shown that this process occurs via facilitation of Na^+/K^+-ATPase activity (Crowe & Hale, 2004) and via adrenergic activation (Crowe et al., 1990). There is also evidence from the rat literature to suggest that BDNF is involved in LTM, and that its function is dependent upon protein synthesis and extracellular signal-regulated kinase (ERK) activity (Alonso et al., 2002; Bekinschtein et al., 2007; Bekinschtein et al., 2008). Note that an effect of BDNF upon one phase of memory does not preclude additional roles existing at other phases. Further experimentation would be useful to systematically determine the time window(s) in which BDNF facilitates memory in the chick, in order to clarify the phase(s) of memory in which BDNF is involved. Doing so on the basis of existing research is problematic due to variations in BDNF manipulations used, species studied and memory tasks employed across experiments.

The saline control data of Experiment 2 are in accord with previous data from our laboratory. Weak training (i.e. 20% v/v MeA), in the absence of memory-enhancing treatments, has been shown to lead to memory for the training event only up until around 35-45 min later. Beyond this point, the memory trace is said to decay (Crowe & Hale, 2002, 2004; Crowe et al., 1989). Similarly, the present study found no significant difference between the saline control and experimental groups until after 40 min following weak training on the PAL task. However, it should be noted that a limitation of the study is the fact that time points between 40 min and 3 hours were not measured.

With regard to Experiment 3 (the reconsolidation study), the results are in contrast to previous work examining BDNF's role in this process. Lee et al. (2004) employed a contextual fear conditioning task in which rats received a foot-shock whilst inside the conditioning chamber. Recall was measured as time spent engaging in freezing behaviour, an indication of fear. Infusion of BDNF antisense ODN 90 min prior to a reminder trial (in which rats were placed in the chamber without the administration of the foot-shock) did not lead to changes in freezing behaviour relative to the BDNF missense ODN control condition. BDNF antisense ODN was, however, effective in blocking consolidation of learned fear when administered 90 min before initial conditioning. Thus, Lee et al.'s (2004) results indicate that while BDNF is required for consolidation of conditioned fear, it is not required for reconsolidation. On the contrary, the current study demonstrated that the application of recombinant BDNF 5 min post-reminder facilitated recall at 3 hours post-reminder relative to saline-injected control chicks, although this effect was not mediated by the time of presentation of the reminder. It is unclear what may account for such a contrasting finding, although a potential explanation is that while BDNF might not be strictly necessary for reconsolidation, its increased presence may nevertheless enhance this process. Evidently, further investigation into the role of BDNF in reconsolidation, beyond the current study and that of Lee et al. (2004), is required.

One methodological concern of the current research is the small sample size used. This may have led to the inconsistency noted between two identical data points in Experiments 1 and 2 (i.e. 50% avoidance for the 12.5 μg/mL data point in Experiment 1 vs. 80% avoidance for the BDNF data point at 180 min in Experiment 2). Thus, where practical, future research would benefit from using larger group sizes.

A further limitation of the present research is that all doses of BDNF employed in Experiment 1 were demonstrated to lead to enhanced memory by increasing avoidance of the red test bead, and no higher dose was used that did not have this effect. Therefore, the

possibility that BDNF caused increased avoidance via a generalised decrease in locomotor behaviour cannot strictly be excluded. Future research should include higher doses of BDNF in order to clarify this issue.

Conclusions

The current study has shown that the application of exogenous BDNF leads to enhancement of memory in the day-old chick. BDNF may be involved at the STM as well as LTM phases of the Gibbs and Ng model of memory, although additional investigation would better clarify this issue. Moreover, BDNF also appears to facilitate memory reconsolidation, but it is unclear whether or not BDNF is strictly necessary for this process to occur.

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Chapter 5: Discussion

In this chapter, a brief summary of the findings of the three studies described in Chapters 2 to 4 is presented. The evidence for the duration of memory retention in the weak training paradigm of the PAL task is discussed, supported by an analysis of the combined control data. An attempt is made to examine the results of all three studies in combination via use of the Gibbs and Ng memory model and via exploration of possible underlying mechanisms of memory formation that may involve a connection between piracetam, memantine, and BDNF. This is followed by a discussion of memory reconsolidation and its biochemical underpinnings in light of the current findings. The chapter concludes with a consideration of the limitations of the present research, suggestions as to future investigations and some final comments.

Summary of Findings

In the current research, each of the three studies began with a dose response experiment, the purpose of which was to determine the most effective dose of the substance under investigation in enhancing passive avoidance learning in the day-old chick, where injection occurred at the time of training and retention was measured at 180 min post-training. Effective doses of piracetam were determined to range from 5 mg/kg (the lowest dose employed) to 25 mg/kg, while higher doses (i.e. 50 and 100 mg/kg) were not effective. This finding is partially consistent with the results of Loscertales, Rose, Daisley and Sandi (1998), who noted memory enhancement in the same task at doses of 10 and 50 mg/kg, but not 100 mg/kg, although note that methodological differences between studies may account for this disparity. Only the 0.1 mM dose of memantine was effective in enhancing memory for the weak training task in the second study, and no dose of memantine (up to 3 mM) affected performance on the strong training task. While no previous investigation has examined memantine's effect in the weak PAL task, the present study's findings in the strong PAL task are

consistent with the findings of Barber, Meyers and McGettigan (2010). Finally, in the BDNF study, all three doses employed led to better retention than the saline condition (6.25, 12.5 and 25 μg/mL), consistent with the results of Johnston, Clements and Rose (1999).

For the piracetam and memantine studies only, a time of injection experiment was carried out to investigate the time window in which administration of the drug led to enhanced memory retention at 180 min post-training. Piracetam led to enhanced memory relative to saline when injected after training, but not at any time before, consistent with Loscertales et al. (1998). Piracetam was effective in facilitating memory even when injected as late as 120 min post-training. Although such a late injection time was not found to be effective by Loscertales et al. (1998), this may be related to the strength of the training event employed. In the memantine study, there was no significant interaction effect between drug injected and time of injection, so comparisons between memantine and saline were not able to be carried out. Nevertheless, memantine led to significantly better retention than saline irrespective of time of injection. Other research in the rat has indicated that memantine is effective when injected prior to but not after training (Wise & Lichtman, 2007).

All three of the studies involved a time of test experiment in order to examine the duration of memory enhancement following administration of each substance immediately after training. In the piracetam study, there was no significant interaction between drug injected and time of test, so comparisons between piracetam and saline at each time of test were not able to be carried out. There was, however, a significant main effect of drug injected, which indicates that piracetam enhanced memory relative to saline overall, regardless of the time of test. The observation of long-term facilitation of memory by piracetam (i.e. at 24 hours post-training) is in keeping with previous findings (Loscertales et al., 1998; Mondadori & Petschke, 1987). For both memantine and BDNF,

there was significant enhancement of memory relative to saline measured from 40 min up until 24 hours (the latest time point observed). Between 10 min and 40 min, there were no differences between memantine and saline, or between BDNF and saline. As a result, it is concluded here that memantine facilitated intact memory, although a large number of previous studies argue that memantine's mode of action is to enhance compromised memory (Barber & Haggarty, 2010; Barber et al., 2010; Beracochea, Boucard, Trocme-Thibierge, & Morain, 2008; Orgogozo, Rigaud, Stöffler, Möbius, & Forette, 2002; Parsons, Stöffler, & Danysz, 2007; Peskind et al., 2006; Winblad, Jones, Wirth, Stöffler, & Möbius, 2007; Winblad & Poritis, 1999). It is suggested that different mechanisms may be involved in memantine's enhancement of intact vs. compromised memory. BDNF's facilitation of memory was in keeping with previous research in the chick (Johnston et al., 1999), and partially in keeping with results from the rodent literature (Alonso et al., 2002).

The last experiment in each study set out to investigate substance effects on memory reconsolidation. This was achieved by administering each substance 5 min following memory reactivation via the presentation of a reminder trial at various times post-training, and memory retention was measured at 180 min post-reminder. For all three studies, there was no significant interaction between drug injected and time of reminder trial. However, all three substances enhanced memory reconsolidation following the reminder relative to saline irrespective of the time of reminder presentation (i.e. a main effect of drug injected was present). While there has been no previous investigation of piracetam's effect upon reconsolidation, the current findings of enhancement of reconsolidation by memantine and BDNF are in contrast to existing research in that memantine has previously been shown to impair reconsolidation (Popik, Wrobel, & Bisaga, 2006), and BDNF has been demonstrated not to be necessary for the process (Lee, Everitt, & Thomas, 2004). However, dose-related differences may explain

the disparity in the case of memantine – Popik et al.'s (2006) study used a much larger dose than that used here, which may have led to inhibition of NMDA receptors such that reconsolidation was inhibited. In the present study, a much smaller dose was used that may have allowed for facilitation. Furthermore, although BDNF may not be strictly necessary for reconsolidation, this does not preclude a non-essential modulatory role.

Weak Training in the PAL Task

The time of test experiments in each of the three studies allowed an examination of weakly reinforced learning in the PAL task without pharmacological manipulation. As described in Chapter 1, the fact that the memory trace in weakly reinforced learning only lasts around 40 min suggests, in the context of the Gibbs and Ng model of memory, that the STM and ITM(A) phases are present, but that ITM(B) and, ultimately, LTM fail to develop (Crowe, Ng, & Gibbs, 1989). The Gibbs and Ng model is discussed further in the following section, but here we compare the duration of memory following weak training in the current studies with previous research from the La Trobe University laboratory.

In the time of test experiments of the memantine and BNDF studies, the results of the saline condition were consistent with previous data of the weak training paradigm from this laboratory. That is, saline-treated chicks avoided the red test bead until 40 min post-training, but after this time, chicks failed to avoid, demonstrating a lack of memory for the training event. However, as reported above, the lack of a significant interaction between drug injected and time of test noted in the piracetam study did not allow the specific examination of avoidance at each time of test. To further confirm the pattern of avoidance over time in the saline condition, data from all three studies were combined and binary logistic regression analysis was carried out, with time of test entered as the categorical independent variable and the avoidance vs. no avoidance binary measure as the dependent variable. This was possible as the control conditions in the time of test

experiments were identical across all three studies. Individual subjects were excluded as per the three original studies (see Chapters 2 to 4). The model was significant ($\chi^2(7) = 44.414$, $p < 0.001$), which justified further analysis at each time of test. Thus, Pearson chi-square tests were carried out to compare the 10 min time point with each of the other time points. As expected, there was no significant difference in avoidance between the 10 and 20 min conditions ($p > 0.05$), but there was a significant difference between the 10 min condition and all other time points (40 min: $\chi^2(1) = 8.525$, $p = 0.004$; 60 min: $\chi^2(1) = 16.258$, $p < 0.001$; 90 min: $\chi^2(1) = 11.267$, $p = 0.001$; 120 min: $\chi^2(1) = 25.535$, $p < 0.001$; 180 min: $\chi^2(1) = 17.779$, $p < 0.001$; 1440 min: $\chi^2(1) = 11.601$, $p = 0.001$). Figure 5.1 illustrates the percentage of chicks in each time of test condition that avoided the test bead. This represents strong support for the duration of memory retention found for weak training in this laboratory (Crowe & Hale, 2002, 2004; Crowe et al., 1989), given the larger sample size used in this analysis.

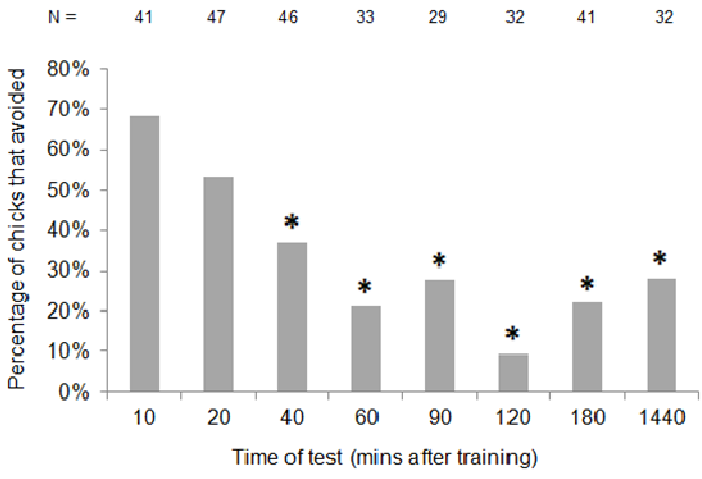

Figure 5.1. Percentage of saline-treated chicks avoiding the red test bead presented at various times post-training, combined across all three studies. Avoidance indicates memory for the weak training event. * = $p < 0.05$ for comparisons of each time-point relative to the 10 min post-training condition.

The Gibbs and Ng Model of Memory

In the preceding chapters, the results of the present research have been explained in the context of the Gibbs and Ng three-phase model of memory formation (Gibbs & Ng, 1976, 1977, 1979; Ng et al., 1997). While the results of the BDNF study are not sufficient to draw conclusions regarding the phase(s) in which this substance may be involved (see Chapter 4), the model is used here to examine the mechanisms of piracetam and memantine in memory, as suggested by the phase(s) of memory upon which these substances appear to have had their effect in the current research.

The findings of the piracetam study indicate that this drug facilitates at least the LTM phase of memory, and may also act at earlier phases in the Gibbs and Ng model. The idea that piracetam facilitates multiple phases of memory supports the idea of a non-specific mechanism for piracetam. For example, Winblad (2005) suggests that piracetam acts at least in part by enhancing cell membrane fluidity. Such an action would not be specific to particular cells or receptor types, and so may account for the facilitation of multiple phases of memory even though these phases may involve different biochemical mechanisms. As mentioned by Winblad (2005), membrane fluidity has been implicated in various cellular processes including receptor binding and activation – processes that are involved in LTP and memory.

On the other hand, it is possible that piracetam facilitates only LTM processes. If this is true, then piracetam's effect at AMPA receptors could certainly account for the memory facilitation seen in the current research. As noted by Ng et al. (1997), LTM can be inhibited by the AMPA receptor antagonist DNQX, which is consistent with AMPA receptor involvement in this phase of memory. Piracetam might alternatively achieve memory enhancement via a positive influence on protein synthesis. This conjecture is based upon the evidence for disruption of LTM processes by protein synthesis inhibitors (Gibbs & Ng, 1977, 1984). However, it is premature to state whether piracetam might

facilitate protein synthesis directly or whether the drug could act upon processes earlier in the biochemical cascade.

Memantine appears to act at either the STM, ITM or both phases of memory in the present research. According to Ng et al. (1997), STM involves neuronal hyperpolarisation dependent on extracellular Ca^{2+} ions. This stage of memory is prevented by administration of a Ca^{2+} channel inhibitor. Thus, assuming an STM-related mechanism for memantine, the drug may act on membrane ion channels in the facilitation of memory formation. However, according to a review by Rogawski and Wenk (2003) memantine acts as a weak inhibitor of Ca^{2+} channels in the hippocampus, so such a mechanism for memantine in enhancing memory may be unlikely. Alternatively, memantine may exert its effect at the ITM phase of memory. Processes suggested to be involved in ITM include Na^{+}/K^{+}-ATPase activity (Gibbs & Ng, 1976; Ng et al., 1997). While memantine has failed to restore reduced Na^{+}/K^{+}-ATPase activity induced by excitotoxic damage in the mouse brain (Chang-Mu, Jen-Kun, Shing-Hwa, & Shoei-Yn, 2010), there is little previous research that has looked at memantine's effects on Na^{+}/K^{+}-ATPase activity under normal, healthy conditions, so conclusions cannot be drawn regarding memantine's impact on this aspect of ITM in the current research. The possibility that memantine may affect STM or ITM via other mechanisms cannot be ruled out at this point in time.

As discussed above, the findings of Chapter 3 indicate that memantine has its effect at the STM and/or ITM phase(s) of memory. By contrast, Ng et al. (1997) note that NMDA receptor activity appears to be involved in the LTM phase. This is consistent with the conclusion drawn in Chapter 3, where the drug was suggested to facilitate memory in the current research via a mechanism not reliant on NMDA receptors.

To conclude, previously proposed mechanisms of action of piracetam have been demonstrated here to be consistent with possible interpretations of this drug's action in

terms of the Gibbs and Ng model of memory in the day-old chick. Regarding memantine, the mechanisms suggested by the model either are inconsistent with existing findings, or have not been widely investigated in previous research. It is worth noting here that the Gibbs and Ng model is by no means an exhaustive description of the biochemical processes underlying memory; this leaves open the possibility that memantine may facilitate memory in the chick in a way that is consistent with a future version of the model.

Piracetam, Memantine and BDNF: Common Mechanisms?

As has been seen, each of the three substances investigated here were demonstrated to facilitate memory in the day-old chick such that they led to the eventual development of LTM. Such consistent findings raise the idea that there could be some relationship between the memory-enhancing mechanisms of these substances. This notion is also in line with existing research, as discussed below.

AMPAkines and BDNF. While there has been no previous research on piracetam's mechanism in connection to BDNF, BDNF has been implicated in the mechanism of action of other AMPAkines. In turn, BDNF has been suggested to play a role in the modulation of AMPA receptor function. The following is an examination of existing evidence in relation to the results of the current studies.

Increases in BDNF mRNA in the rat hippocampus *in vitro* and *in vivo* have been demonstrated to occur following direct agonist action at AMPA receptors (Zafra, Hengerer, Leibrock, Thoenen, & Lindholm, 1990). These researchers reported that this effect was selectively inhibited by AMPA receptor antagonist CNQX, and not NMDA receptor antagonist MK-801. In line with this finding, AMPAkines, which do not have a direct agonist action but positively modulate AMPA receptors, have been shown to have similar effects on BDNF mRNA levels. Lauterborn, Lynch, Vanderklish, Arai and Gall (2000) found that acute treatment with AMPAkines CX614 and CX546 dose-dependently

led to increased expression of BDNF mRNA in rat hippocampi *in vitro*. They noted that the CX614-mediated increase was abolished following treatment with CNQX, but not with NMDA receptor antagonist AP5, confirming the role of AMPA receptors in this process. CX614 was also able to increase levels of BDNF protein *in vitro*, and CX546 was shown to increase BDNF mRNA expression in aged rats and middle-aged mice *in vivo*. Additionally, Mackowiak, O'Neill, Hicks, Bleakman and Skolnick (2002) determined similar dose-dependent effects of the AMPAkine LY451646 on BDNF mRNA in rat hippocampi. They further found an increase in BDNF protein resulting from treatment with LY404187 *in vivo*. These findings are consistent with the idea that piracetam may influence BDNF via its action at AMPA receptors, although further work is needed to directly investigate the link between piracetam and BDNF.

Some of the effects described above were noted to occur with some regional specificity within the hippocampal formation. For example, acute and chronic LY451646 treatment led to increased BDNF mRNA expression in the dentate gyrus and areas CA3 and CA4 of the hippocampus, but not in the CA1 region (Mackowiak et al., 2002). This parallels piracetam's effects on hippocampal LTP described in Chapter 1 – namely, that piracetam facilitates LTP at synapses in the CA3 region (He, Liao, Zheng, Zeng, & Guo, 2008; Satoh, Ishihara, & Katsuki, 1988; Sugimura, Ishihara, Katsuki, & Satoh, 1989), and not in other regions such as CA1 (Chepkova et al., 1995; Satoh et al., 1988). In contrast, other AMPAkine-related effects on BDNF expression were specific to the dentate gyrus only, another area in which piracetam has reportedly failed to facilitate LTP (Molnár & Gaál, 1992), although differences in effect across AMPAkines are not unexpected.

On a functional level, an AMPAkine-mediated increase in BDNF has been demonstrated to restore impaired LTP in middle-aged rat hippocampi *in vivo* (Rex et al., 2006). These authors noted that administration of CX929, which has a half-life in rats of

15 min, led to increased hippocampal BDNF expression measured almost a day after the final injection, long after the drug would have been metabolised and/or eliminated. They also found that CX929 prevented the decay of LTP in the same hippocampal slices in which increased BDNF was observed, and that this effect on LTP was abolished by a treatment which removes extracellular BDNF. Rex et al. (2006) concluded that increasing endogenous levels of BDNF via AMPAkine treatment leads to stabilisation of LTP. In light of the assumed link between LTP and memory formation, these results provide a mechanism by which AMPAkines such as piracetam may influence memory. Further research is needed to confirm whether or not a mechanistic link between piracetam and BDNF is associated with memory function at the behavioural level.

A review by O'Neill and others describes the proposed mechanisms behind AMPAkine-mediated increases in BDNF (O'Neill, Bleakman, Zimmerman, & Nisenbaum, 2004). Evidence exists for two pathways: one involves activation of the MAPK cascade by the tyrosine kinase Lyn which is physically associated with AMPA receptors (non-activity-dependent); the other involves an increase in intracellular Ca^{2+} via the opening of voltage-gated Ca^{2+} channels, triggered by AMPA receptor-mediated membrane depolarisation (activity-dependent). Both pathways lead to increased BDNF expression. O'Neill et al. (2004) suggest that both pathways may contribute to the effect, given that: (a) inhibiting either pathway in isolation leads to a partial reduction in an AMPAkine-mediated increase in BDNF mRNA, and; (b) inhibiting both pathways simultaneously leads to complete abolition of the BDNF mRNA increase (Legutko, Li, & Skolnick, 2001).

Whereas the above data constitute compelling evidence for the influence of AMPA receptor modulation on BDNF, other research suggests that BDNF indirectly impacts upon AMPA receptor function. As described in Chapter 1, Baudry and colleagues argue that the maintenance of LTP involves reorganisation of the actin cytoskeleton of

the synapse via activation of calpain (Baudry, Bi, Gall, & Lynch, 2011; Lynch & Baudry, 1984). Such morphological changes to the synapse allow for the insertion of new AMPA receptors, an event involved in LTP expression (Malinow & Malenka, 2002). The role of BDNF in this cascade of cellular events is to bring about the activation of calpain via an ERK-mediated mechanism (Zadran et al., 2010). Furthermore, BDNF has been shown to increase AMPA receptor protein levels in neocortical neurons in culture (Narisawa-Saito, Carnahan, Araki, Yamaguchi, & Nawa, 1999). These findings point toward mutual modulation between BDNF and AMPA receptors.

Thus, in explaining how both the AMPAkine piracetam and BDNF lead to enhancement of memory consolidation in the present research, it is possible that related pathways at the cellular level were involved. As described above, AMPAkines in general have the potential to increase levels of BDNF mRNA and BDNF protein, although it remains to be determined whether or not piracetam in particular can influence BDNF levels in the brain. In turn, BDNF plays a role in the expression and maintenance of LTP such that AMPA receptor insertion, a marker of LTP expression, is made possible. The link between LTP and memory suggests that these events may underlie memory formation.

Memantine and BDNF. Previous research has shown that memantine can influence BDNF expression. Also, BDNF/TrkB receptor activity has been demonstrated to influence NMDA receptor function. Mechanisms for memantine's effect on BDNF-related factors are discussed here in light of present findings, including an exploration of mechanisms not involving NMDA receptors.

There are several lines of evidence that point toward a facilitatory effect of memantine upon BDNF expression. Memantine has been specifically shown to increase levels of BDNF mRNA in various areas of rat brain slices at the clinically relevant dose of 5 mg/kg (Marvanová et al., 2001). Higher doses (25 and 50 mg/kg) led to increases in

BDNF mRNA in the hippocampal formation, and BDNF protein was seen to increase in the retrosplenial cortex following treatment with 50 mg/kg of memantine, although other brain regions were not analysed for this measure. However, animals injected with the higher doses were observed to appear sedated, and so the effects of these higher doses were suggested to reflect neurotoxic processes. Réus et al. (2010) noted that acute injection of 20 mg/kg memantine increased levels of BDNF protein in the rat hippocampus, while neither lower acute doses (5 and 10 mg/kg) nor chronic administration had any effect.

On the other hand, chronic memantine treatment has been demonstrated to increase hippocampal BDNF mRNA and cortical BDNF protein in a mouse model of Down syndrome (Lockrow, Boger, Bimonte-Nelson, & Granholm, 2011) and to increase BDNF protein levels in the hippocampus and cortex in healthy mice (Molinaro et al., 2009). Finally, rhesus monkeys infected with simian immunodeficiency virus and treated with chronic memantine were found to have greater BDNF protein levels relative to both untreated and uninfected controls (Meisner et al., 2008). However, the dose of memantine used here (0.1 mM, approximately equivalent to 0.048 mg/kg) differs markedly from even the lowest acute dose used by Marvanová et al. (2001; 5 mg/kg). This could mean that the current dose of memantine used was not high enough to influence BDNF expression, or that the responsivity of the chick is different to these other species.

Regarding BDNF-dependent effects, Mizuno, Yamada, He, Nakajima and Nabeshima (2003) recently demonstrated that BDNF/TrkB activity is associated with enhanced NMDA receptor function in rats via a tyrosine kinase called Fyn. They noted increased phosphorylation of Fyn and of the NMDA receptor subunit NR2B (but not NR2A) in the hippocampus following spatial learning. This effect is in keeping with previous research in which phosphorylation of NR2B (and not NR2A) resulted from

treatment with BDNF in cortical and hippocampal post-synaptic densities (Lin et al., 1998). Mizuno et al. (2003) also found that Fyn was significantly associated with TrkB and NR2B via immunoprecipitation following spatial memory formation, and that administration of PP2 (an inhibitor of tyrosine kinases, including Fyn), led to both impaired spatial learning and reduced phosphorylation of Fyn and NR2B. Additionally, Suen et al. (1997) noted that application of BDNF increased the phosphorylation of the NR1 subunit in hippocampal synaptoneurosomes and post-synaptic densities. These studies indicate that BDNF may influence memantine's NMDA receptor-mediated effect upon memory.

However, memantine's effect as outlined in Chapter 3 may involve a mechanism that is not dependent on NMDA receptors. Chapter 1 describes memantine's action in terms of the theory put forward by Parsons et al. (2007). This theory suggests that memantine counteracts pathological glutamatergic activity while sparing physiological activity of the NMDA receptor. This mechanism, therefore, requires dysfunctional glutamatergic activity to be present (e.g. as a result of age-related decline, stress, pharmacological manipulations, etc.) in order for memantine's effect to be apparent, whereas it is argued in Chapter 3 that no such condition applied to the young, healthy, non-stressed chicks employed in the present research. This could mean that some other mechanism not involving NMDA receptor function may explain the memantine-mediated facilitation of memory seen in Chapter 3. It should be noted that this proposal does not negate the existence of Parsons et al.'s (2007) mechanism; rather, it attempts to account for memantine's action under a different set of circumstances.

One potential mechanism for memantine discussed in Chapter 3 was positive modulation of muscarinic acetylcholine (ACh) receptors. Interactions between these receptors and BDNF/TrkB activity have been linked to memory formation. For example, Knipper et al. (1994) have shown that muscarinic receptor activation increases the

production of BDNF mRNA in the rat hippocampus. Given the established role of the hippocampus in memory, the interaction between muscarinic receptor function and BDNF may reflect a possible memory mechanism. Additionally, Gil-Bea et al. (2011) demonstrated that cholinergic denervation in the rat hippocampus results in decreased expression of BDNF as indicated by reduced levels of proBDNF, the precursor of BDNF. It was observed that the reduction of proBDNF could be rescued by a muscarinic receptor agonist, supporting a link between this specific receptor type and BDNF activity. It was also found that both proBDNF levels and performance on a spatial memory task were reduced relative to the intact learning observed in sham-operated control rats, and that further training trials led to both improved memory and increased proBDNF levels such that there were no significant differences relative to controls. Assuming a muscarinic receptor-mediated mechanism of action for memantine, Gil-Bea et al.'s (2011) findings support the notion that memantine may have influenced memory in chicks in the current research via increasing BDNF levels.

Memantine's effect in the current research may alternatively have been the result of antagonistic action at serotonin (5-HT) receptors – specifically, the 5-HT_3 receptor. This receptor is a ligand-gated ion channel, unlike other 5-HT receptors which are G protein-coupled metabotropic receptors. 5-HT_3 receptors also bind 5-HT with the lowest affinity of all 5-HT receptors (Uphouse, 1997). This factor may explain why it is possible that inhibition of 5-HT_3 receptors facilitates memory (Barnes et al., 1990; Chugh, Saha, Sankaranarayanan, & Sharma, 1991; Pitsikas, Brambilla, & Borsini, 1994), while the action of 5-HT positively influences memory overall (e.g. Olivier et al., 2009). With regard to a combined mechanism between BDNF and memantine via 5-HT receptors, Mattson, Maudsley and Martin (2004) argue that 5-HT appears to act cooperatively with BDNF in the regulation of neuronal plasticity. However, there is currently no research that specifically addresses the relationship between 5-HT_3 receptor

function and BDNF/TrkB activity, so for now, assuming a 5-HT_3 receptor-mediated mode of action of memantine in the present research, a link between memantine and BDNF can only be speculative.

In conclusion, it is unclear whether memantine's effect in the present research involved a BDNF-related mechanism. While memantine certainly has been demonstrated to influence BDNF expression previously, the cellular pathway by which this process occurs as yet has not been clearly elucidated. Furthermore, the mechanism by which memantine facilitated memory in the present research has also not been confirmed. It is, however, certainly feasible that the effect of BDNF may have incorporated an NMDA receptor-dependent mechanism in addition to the AMPA receptor-related function described above, although as stated, it is unclear whether this mechanism or another relates to memantine's action in the current research.

Memory Retrieval and Reconsolidation

Before a memory can be reconsolidated, it must first be retrieved from storage. Not only does the reminder paradigm employed in the current series of experiments elicit reconsolidation, it prompts the chick to recall the memory associated with the learning event. Previous research has examined the effect of memory retrieval in the chick upon later retention of the weak PAL task. Summers, Crowe and Ng (2000) noted that presentation of a reminder trial could strengthen memory for a weak training event such that retention was the same as that for the strong version of the task. Since the weak PAL task in combination with a reminder trial was employed in the present research, the saline control data from all three studies are combined here in order to explore the pattern of retention following reminder.

A binary logistic regression analysis was carried out, and the model was non-significant ($p > 0.05$), indicating no effect of varying the time of presentation of the reminder trial. Figure 5.2 depicts the percentage avoidance of the test bead for each

condition. Percentage avoidance is low across all groups (a mean of 25% avoidance), which represents poor recall of the training event. This suggests that the presentation of a reminder trial in the absence of other experimental manipulations did not enhance memory for the weak training stimulus. Indeed, the average percentage avoidance following reminder is comparable to levels of avoidance seen from 60 minutes and later in the absence of a reminder trial (see Figure 5.1). This finding is not consistent with previous research from this laboratory. It has been reported that a reminder trial, when presented 7.5 or 25 min following weak training (i.e. during either the STM or ITM(A) phases of memory), enhances memory such that testing at 180 min post-training reveals a retention level equivalent to that found following strong training (Summers et al., 2000). Reminders presented at 10 and 20 min post-training did not have this effect. In contrast, reminder presentation at 40 or 120 min (i.e. during the ITM(B) or LTM phases) did not lead to enhancement of the weakly learned memory in Summers et al.'s (2000) study. Data from similar groups in the present analysis (40, 60, 180 and 1440 min) are therefore in line with previous findings.

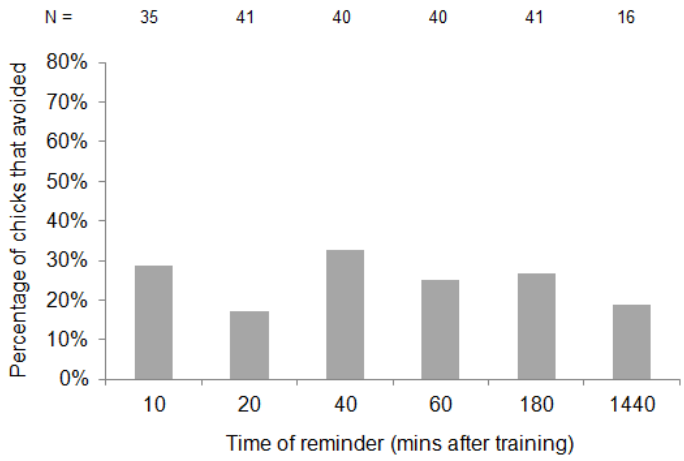

Figure 5.2. Percentage of saline-treated chicks avoiding the red test bead presented at 180 min post-reminder, combined across all three studies. Presentation of the reminder trial occurred at various times post-training, and avoidance indicates memory for the weak training event.

Based on their previous research in the day-old chick, Summers, Crowe and Ng (2003) have suggested that there are two distinct processes that follow retrieval of a memory. The first is a brief phase that may function as an immediate recall mechanism. This phase is sensitive to disruption by the application of the Ca^{2+} channel blocker lanthanum chloride, with retention deficits resolving within 10 to 15 min following the reminder trial. The second phase appears to be dependent on the function of glutamate and lasts around 24 hours post-reminder. It is this second phase at which the substances applied in the current research are likely to have had their respective effects, given that facilitation of memory was noted 180 min post-reminder in all three studies. Additionally, this explanation is consistent with Summers et al.'s (2003) model from a biochemical viewpoint. For example, piracetam, via action at AMPAergic glutamate receptors, may positively modulate this glutamate-sensitive phase, given that blockade of AMPA receptors by CNQX has previously been shown to interfere with reconsolidation (Sherry & Crowe, 2008). Moreover, it may be argued that BDNF affected the second phase of reconsolidation when taking into consideration the downstream effects of AMPA receptor modulation discussed in the previous section.

In sum, the current research did not find enhanced retention following retrieval of the weak PAL memory trace during the early phases of memory, in the absence of pharmacological manipulation. The memory-enhancing agents used in the present series of studies appeared to have their impact upon Summers et al.'s (2003) second phase of reconsolidation.

Limitations of the Present Research

One of the methodological concerns that must be addressed here is the variation of the time of day at which bead pecks were recorded. Some of the experimental conditions involved delays which meant that test trials took place quite late in the day and many chicks were anecdotally observed to be difficult to rouse at the time of test

trials. Indeed, a number of birds failed to open their eyes at all during the 10-second presentation of the red bead upon testing, and this may have led to a higher percentage of chicks recorded as avoiding the bead in groups tested late in the day. The time of day at which each group of chicks was presented with the test trial is displayed in Figure 5.3. As can be seen, there was indeed substantial variation with respect to the time of day at which testing took place in some of the experiments. In particular, the time of reminder trial experiments (Figure 5.3, circles) led to test trials occurring late in the day for some groups, which meant wider variation in test trial times. There was also substantial variation in test trial times in the time of test experiment with BDNF (Figure 5.3, grey triangles).

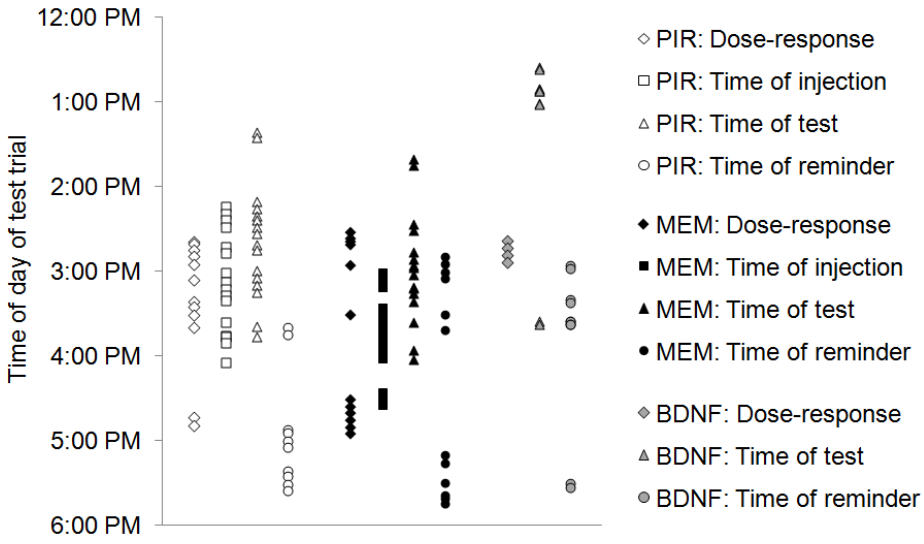

Figure 5.3. Distribution of PAL test trial times occurring for each experiment. Each marker represents one experimental group of chicks (i.e. 20 subjects), and the position of each marker indicates the time of day at which the test trials began for that group. PIR = piracetam, MEM = memantine, BDNF = brain-derived neurotrophic factor.

In support of this notion, it has been shown empirically that chicks are subject to diurnal variation in performance on learning tasks (Radford, Ng, & Armstrong, 1981; Reymond & Rogers, 1981). Radford and colleagues used chicks that had been trained on the discrimination variant of the PAL task at 8am, 12pm, 4pm and 8pm and tested at the same respective time on the following day. For control chicks trained with water-coated red and blue beads, the authors found that chicks trained at 8pm and tested at 8pm 24 hours later had a greater tendency to avoid the test beads relative to those trained at earlier time points. Variation in memory retention with respect to time of day was also noted in the experimental groups (i.e. trained with a MeA-coated red bead and a water coated blue bead). Chicks trained and tested at 4pm were noted to display higher levels of generalised avoidance – that is, they avoided both the trained and untrained beads (Radford et al., 1981). Therefore, concerns about performance variation with time of day appear to be warranted, and to avoid a potential confound in subsequent experimentation, it is suggested that testing occurs at a consistent time of day where practicable.

A further limitation of the present research is the small group size used in the BDNF experiments. A group size of 10 was employed due to financial constraints, as opposed to the 20 chicks per group used in the piracetam and memantine studies. As noted in Chapter 4, the small sample size may have been the cause of an inconsistency in results between two identical experimental groups. It is acknowledged that group sizes of at least 20 subjects are preferable, in order to increase power and to decrease the likelihood of obtaining false-positive results.

Other inconsistencies in results, both between studies in the current research and between this and previous research, may be at least partially explained by the use of the binary dependent variable. Previous chapters have outlined the justification of the choice of the dependent measure; that is, the violation of parametric assumptions by the previously chosen continuous measure. However, it must be acknowledged that the

collapsing of this measure into binary form necessarily resulted in loss of information and reduced sensitivity in the analyses, which may have had some impact upon the results in the form of increased likelihood of false-negative errors.

Additional limitations of the experimental methodology are as follows:

- Lack of water-coated control bead used in training – this can lead to difficulty in ruling out confounds such as non-specific avoidance of the test bead.
- Use of a subcutaneous route of administration of piracetam and memantine – this does not allow for regional specificity of the drug target.
- Use of the day-old chick as a model – this may not allow for straightforward generalisation of results to the mammalian memory system.
- Lack of biochemical assays – this meant that the effect of the substances could not be directly verified.

Directions for Future Research

While a number of AMPAkines have been shown to involve a BDNF-related mechanism, such a mechanism has not been demonstrated for piracetam in particular. Thus, a biochemical study of the effect of piracetam on BDNF mRNA and protein levels would be useful in order to determine whether the mechanism of this AMPAkine is similar in nature to that of other AMPAkines.

Further research on mechanisms of memantine is also warranted. In particular, there is limited understanding of the ways in which memantine may act: (a) that do not involve the NMDA receptor, and; (b) that may stem from the application of very low doses. The study described in Chapter 3 has indicated that memantine can improve healthy memory – the underlying mechanism of this process may be different to the one suggested by Parsons et al. (2007), and further investigation would help to shed light on this mechanism.

An experiment similar to the time of injection experiments used here with piracetam and memantine would be useful to examine effects of BDNF administration. This would provide further evidence for BDNF's effects in terms of the Gibbs and Ng model of memory. Unfortunately, financial limitations prevented the inclusion of such an experiment in the present study.

Regarding memory reconsolidation, there is a call for further examination of the roles of each of piracetam, memantine and BDNF in this process given the dearth of existing evidence. Future investigations should use different subjects and different memory tasks in an effort to demonstrate whether or not the current findings are able to be generalised beyond the paradigm employed here. Additionally, the reconsolidation experiment employed here may be expanded upon to include extra control groups as follows: drug with reminder; saline with reminder; drug with no reminder; saline with no reminder. Comparisons between each of the four conditions could be made for reminders (or injections without reminders) presented at various times following training, as in the current study. These comparisons would represent an interesting addition to the current findings and would serve to further confirm the results reported here.

Concluding Remarks

This thesis has demonstrated that piracetam, memantine and BDNF can enhance memory consolidation and reconsolidation in the day-old chick, as measured using the PAL task. It has also been suggested that the cellular processes involved in memory facilitation by these substances are interconnected. While the details of the mechanism(s) behind these behavioural findings remain to be elucidated, the current research has allowed for new questions to be asked about the biochemical processes of memory in the chick.

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