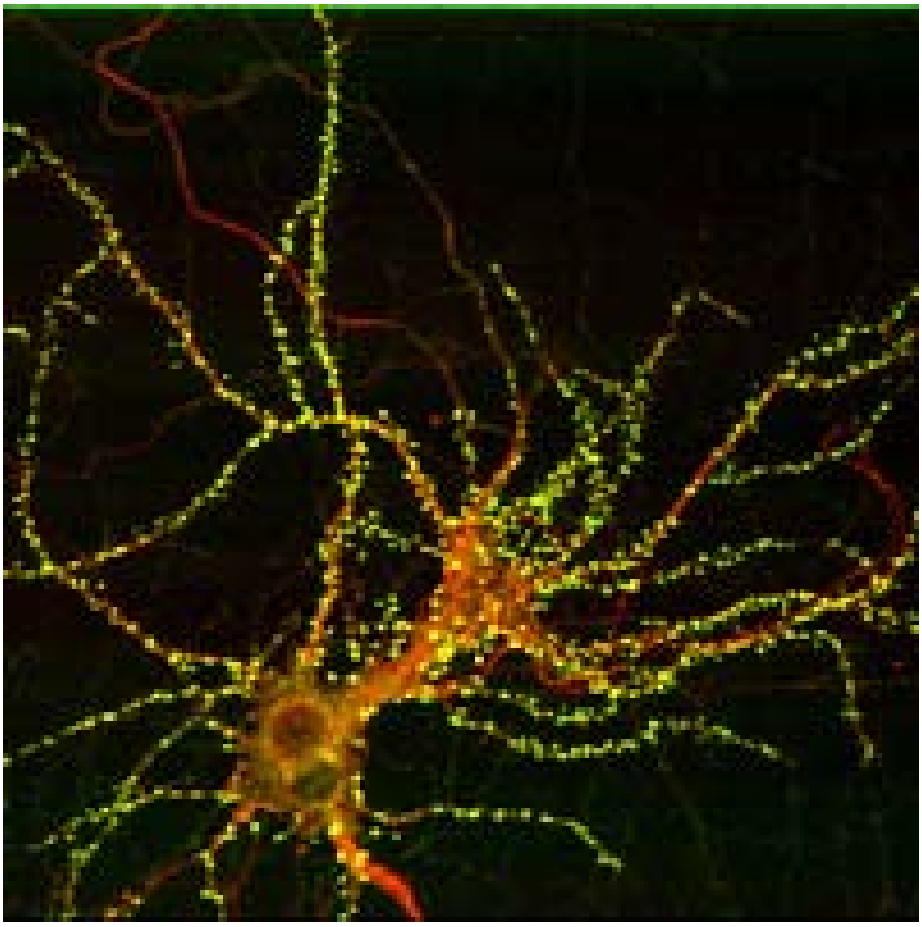
The Role of Spinophilin in Morphine Addiction



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Abstract

Spinophilin (SPL), also termed Neurabin II, was initially discovered as a protein phosphatase 1 (PP1) binding protein and was first cloned in 1997 [Allen PB et al, 1997]. SPL, although ubiquitous, was found to be highly expressed in dendritic spines [Feng et al, 2000], suggesting that it has a specific involvement in the regulation of excitatory transmission. Since then SPL has been shown to bind to a growing number of proteins. Due to spinophilin enrichment at dendritic spines, and for its choice of partners, it has been hailed as having a major role in regulation of excitatory synaptic transmission, signal transduction, dendritic spine formation and plasticity. The role that SPL plays in the adaptive process underlying opiate addiction is not fully understood.

The present study examined, by western blot analysis, the regulation of SPL levels in the nucleus accumbens (NAc) following acute and chronic morphine administration in mice. Following acute morphine administration, SPL levels appeared to be decreased compared to saline treated animals at both 10 minutes and 2 hours post injection. Following chronic morphine exposure the level of SPL protein appeared to be significantly increased. These results demonstrate that SPL is regulated in vivo after morphine administration and SPL plays a role in the adaptive molecular changes associated with opiate addiction.

The development of dependence and tolerance to morphine is the major limiting factor to the treatment of chronic pain with the opiate. It has been previously reported that morphine, which binds solely to Mu family of opiate receptors (MOR), does not readily induce receptor endocytosis and this factor contributes to the development of tolerance. Confocal microscopic analysis in transiently transfected HEK293 cells revealed that in the presence of SPL, MOR was internalized after a 10min treatment with morphine but remained on the surface in the absence of SPL. These observations suggest that SPL

plays and important role in GPCR endocytosis, the development of tolerance, and the addictive state. Interestingly, GFP-tagged SPL was also found to be translocated to the cytosol after MOR agonist treatment in PC12.

The present data suggest an essential role of SPL in MOR functional responses, and in the adaptive changes associated with opiate addiction.

Περίληψη

Η spinophilin (SPL) ή Neurabin II ανακαλύφθηκε σαν μια από τις πρωτεΐνες που αλληλεπιδρούν με την Πρωτεϊνική Φωσφατάση 1 και κλωνοποιήθηκε για πρώτη φορά το 1997 [Allen PB et al, 1997]. Αν και εντοπίζεται παντού στο Κεντρικό Νευρικό Σύστημα, η SPL εκφράζεται σε μεγάλο βαθμό στις δενδριτικές άκανθες [Feng et al, 2000], γεγονός που υποδηλώνει ότι πιθανώς έχει σημαντικό ρυθμιστικό ρόλο στην μετάδοση κυτταρικής σηματοδότησης. Πρόσφατες μελέτες έδειξαν ότι η SPL προσδένεται σε ένα μεγάλο αριθμό πρωτεϊνών. Η αλληλεπίδραση της με τις πρωτεΐνες αυτές και ο εντοπισμός της στις δενδριτικές άκανθες, κάνουν την SPL έναν από τους πιο σημαντικούς ρυθμιστές της κυτταρικής σηματοδότησης αλλά και του σχηματισμού και πλαστικότητας των δενδριτικών ακάνθων. Ο ρόλος της SPL στην προσαρμοστική διεργασία που χαρακτηρίζει τον εθισμό στα οπιοειδή δεν έχει ακόμα κατανοηθεί πλήρως.

Στην παρούσα εργασία εξετάστηκε, με ανάλυση ανοσοαποτύπωσης, η ρύθμιση των επιπέδων της SPL στον επικληνή πυρήνα (NAc) μετά από οξεία και χρόνια χορήγηση μορφίνης σε ποντίκια. Μετά από οξεία χορήγηση μορφίνης τα επίπεδα της SPL φάνηκε να μειώνονται, σε σύγκριση με αυτά που παρατηρούνται σε ζώα που τους είχε χορηγηθεί αλατούχο διάλυμα, 10 λεπτά αλλά και 2 ώρες μετά από τις ενέσεις. Μετά από χρόνια έκθεση σε μορφίνη, το επίπεδο της SPL εμφανίζεται σημαντικά αυξημένο. Αυτά τα αποτελέσματα δείχνουν ότι η SPL ρυθμίζεται in vivo μετά την χορήγηση μορφίνης και ότι παίζει ένα ρόλο στις προσαρμοστικές μοριακές αλλαγές που συνδέονται με τον εθισμό σε οπιοειδή.

Η ανάπτυξη εξάρτησης και ανθεκτικότητας στην μορφίνη είναι ο μεγαλύτερος περιοριστικός παράγοντας στην θεραπεία χρόνιου άλγους με οπιοειδή. Όπως έχει αναφερθεί σε προηγούμενες μελέτες, η μορφίνη, η οποία συνδέεται μόνο με τον μ υποδοχέα οπιοειδών (MOR), δεν προκαλεί άμεση ενδοκύτωση του υποδοχέα και το γεγονός αυτό συνεισφέρει στην ανάπτυξη ανθεκτικότητας. Η ανάλυση παροδικά επιμολυσμένων κυττάρων HEK293 με χρήση συνεστιακής

μικροσκοπίας αποκάλυψε ότι παρουσία της SPL οι MOR είχαν ενδοκυτταρωθεί μετά από μια δεκάλεπτη χορήγηση μορφίνης αλλά παρέμειναν στην επιφάνεια όταν η SPL ήταν απούσα. Αυτές οι παρατηρήσεις οδηγούν στο συμπέρασμα ότι η SPL παίζει ένα σημαντικό ρόλο στην ενδοκύτωση των GPCR, στην ανάπτυξη ανθεκτικότητας και την εθιστική κατάσταση. Είναι επίσης ενδιαφέρον ότι η GFP-tagged SPL βρέθηκε να είναι μετατοπισμένη στο κυτοσόλιο μετά την επίδραση αγωνιστών των MOR σε κύτταρα PC12.

Τα αποτελέσματα που παρουσιάζονται σε αυτή την εργασία οδηγούν στο συμπέρασμα ότι η SPL έχει ένα ουσιώδη ρόλο στην ρύθμιση της ανταπόκρισης των υποδοχέων των μ οπιοειδών (MOR) και στις προσαρμοστικές αλλαγές που σχετίζονται με τον εθισμό σε οπιοειδή.

1. INTRODUCTION

1.1 Introduction

Spinophilin (SPL) was discovered as a protein phosphatase 1 (PP1) binding protein and cloned by two independent research teams in 1997 [Allen et al., 1997] and in 1998 [Satoh A et al., 1998]. SPL was found to be highly expressed in dendritic spines, the site for excitatory transmission. Spinophilin has been shown to bind to a growing number of proteins including its homologue termed neurabin 1, phasphatases [Allen et al., 1997, Satoh et al. 1998] kinases, RGS proteins [Wang et al. 2005], GPRC [Allen et al., 1997, Yan et al., 1999, Feng et al., 2000, Smith et al 1999], actin cytoskeleton [Satoh et al., 1998] and antagonizes arrestins [Wang et al., 2004]. There is an emerging role for SPL in opiate addiction.

1.2 Pathways Involved in Opiate Addiction.

Drug addiction can be defined as a chronic, relapsing brain disorder characterized by neurobiological alterations leading to compulsive drug seeking and taking behavior [Kaye et al, 2003]. After long term use of opiates the nerve cells, normally producing endogenous opiates, cease to function and degeneration causes the user to become physically dependent on exogenous opiates. There are 4 key stages associated with drug action; 1) acute drug action: reinforcement/reward, 2) Chronic drug action: tolerance, sensitization, dependence, 3) short term abstinence: withdrawal, 4) Long term abstinence: craving, stress-induced relapse [Nestler, 1997].

Morphine is one of the most commonly used drugs in the treatment of severe and chronic pain. A major complication with its long term use is that patients develop tolerance and dependence to the drug [Haberstock-Debic H et al., 2003, Nestler 2001]. The development of morphine tolerance occurs through continued use of the drug such that the amount of drug required to elicit pain

relief must be increased to compensate for diminished responsiveness. A way of reducing tolerance would be of great benefit, as it would allow doctors to prescribe lower doses over longer periods while still effectively controlling pain.

Knowledge of the neural mechanisms that underlie the change from casual drug use to addiction is still incomplete. However, it is known that drugs are abused due to their initial intense rewarding effects. Drugs induce alterations in neurotransmitter and neuropeptide systems that regulate incentive-motivation [Ammon-Treiber et al., 2005]. One such system is the mesoaccumbens dompamine system that projects from the ventral tagmented area (VTA) to the nucleus accumbens (NAc) with afferent and efferent projections to the medial prefrontal cortex (mPFC), amygdala, and ventral pallidum (see fig 1.1).

1.3 G Protein Coupled Receptors (GPCR) in Morphine Addiction.

G protein coupled receptors (GPCR) constitute a superfamily of seven transmembrane spanning proteins that respond to a diverse array of stimuli such as hormones and neurotransmitters. GPCRs transmit the information provided by these stimuli into intracellular secondary messages. This process involves the linking of agonist activated GPCRs to a wide variety of intracellular signalling pathways via their interaction with heterotrimeric guanine nucleotide binding proteins, or more simply, G proteins. G Proteins are composed of 3 subunits α, β and γ. The binding of the agonist to GPCR selects for the receptor conformation state that prompts the exchange of GDP to GTP on the α subunit and allows for the disassociation of the Gα subunits from Gβγ. Subsequently, the activated subunits positively and/or negatively regulate the activity of effector systems (for comprehensive review, refer to Ferguson 2001, also see fig, 1.2 Schematic diagram of G protein function).

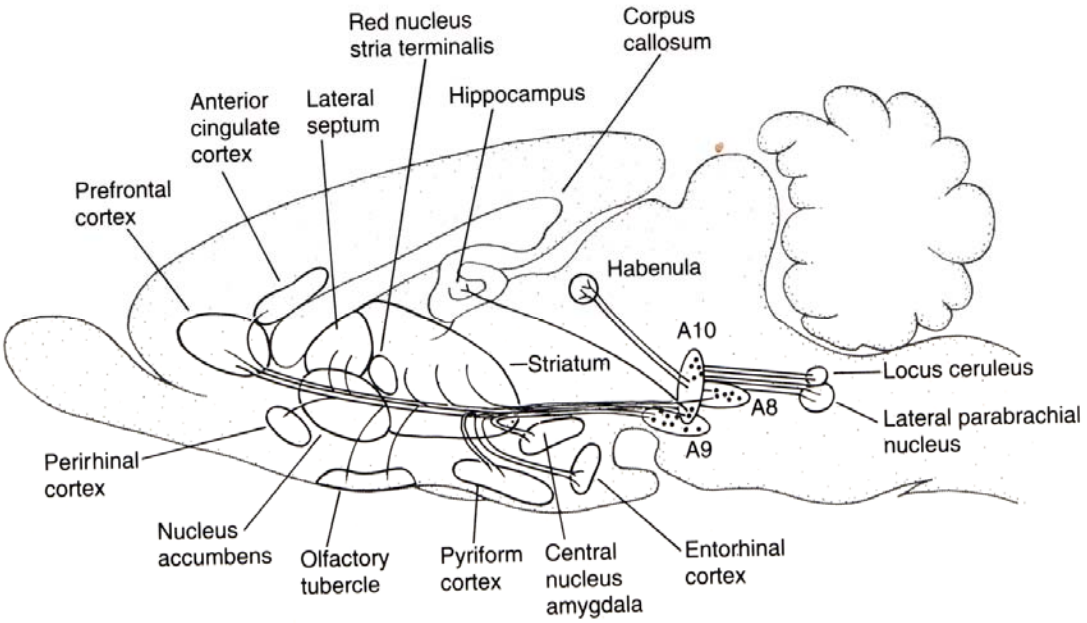

Figure 1.1 Two main dopaminergic cell groups with in the midbrain. The Dopaminergic system originates in the midbrain and projects to the striatum, limbic system and Neocortex. There are two main cell groups, (1) the mesostriatal system and (2) The mesolimbic and mesocortical systems. The first system projects from the substantia nigra and the ventral tagmented area VTA (Nucleus 10) to several striatal areas including the nucleus accumbens (Nac) and is primarily involved in the control of voluntary movement as well as the rewarding effects of drugs of abuse. The second system projects from the VTA to limbic and cortical areas including the locus ceruleus (LC) the major Noradrenergic centre of the brain. This system is believed to be the primary reinforcing pathway for drugs of abuse. **Taken from Principals of Neuroscience, Third edition, ER Kendal (1991)**

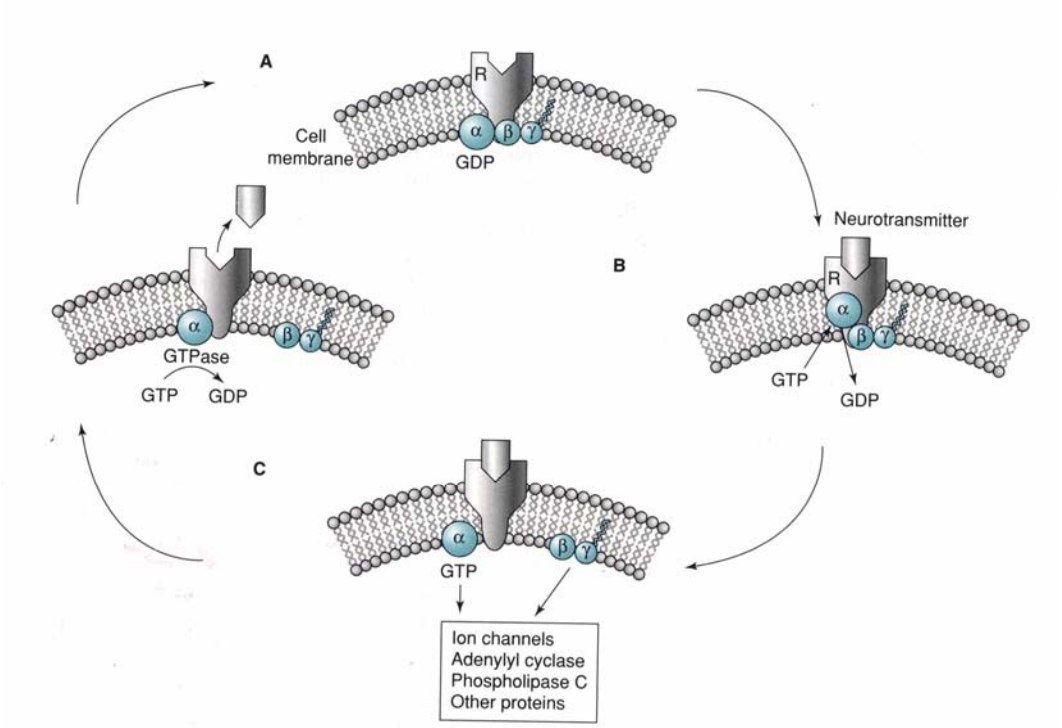

Figure 1.2- G protein function. **A**. Under basal conditions, G proteins exists in cell membranes as hetrotrimers composed of single α, β and γ subunits, The a subunits are bound to the GDP and G protein is anchored to the plasma membrane. **B.** After receptor (R) is activated by its ligand it associates with the α subunit causing the later to release bound GDP. Subsequent GTP binds to the α subunit. **C.** GTP binding causes the disassociation of the a subunit from the βγ subunits and from the receptor. Both the α subunits, bound to GTP, and the βγ subunit, are functionally active and directly regulate a variety of proteins including ion channels and down stream effector molecules **D.** GTPase intrinsic activity degrades GTP to GDP, and in turn causes reassociation of the α and βγ subunits. This reassociation, in conjunction with the disassociation of the ligand from the receptor, restores the receptor to the basal state. **Taken from Molecular Neuropharmacology. A foundation for Clinical Neuroscience. E J Nestler 2001.**

Three major types of G proteins are involved in signal transduction produced by neurotransmitter binding, Gs, (stimulatory) G i/o, (inhibitory) and Gq. See table 1 below for details of their actions.

Class	Molecular Mass (kDa)	Toxin-Mediated ADP-Ribosylation	Effector Protein(s)
G_s family			
$G_{\alpha s1}$	52	Cholera	Adenylyl cyclase (activation)
$G_{\alpha s2}$	52		
$G_{\alpha s3}$	45		
$G_{\alpha s4}$	45		
$G_{\alpha olf}$	45		
G_i family			
$G_{\alpha i1}$	41	Pertussis	Adenylyl cyclase (inhibition)
$G_{\alpha i2}$	40		?K^+ channel (inhibition)
$G_{\alpha i3}$	41		?Ca^{2+} channel (activation)
			?Phospholipase C (activation)
			?Phospholipase A_2
$G_{\alpha o1}$	39	Pertussis	?K^+ channel (inhibition)
$G_{\alpha o2}$	39		?Ca^{2+} channel (activation)
$G_{\alpha t1}$	39	Cholera and pertussis	Phosphodiesterase in rods and cones (activation)
$G_{\alpha t2}$	40		
$G_{\alpha gust}$	41	Unknown	Phosphodiesterase in taste epithelium (activation)
$G_{\alpha z}$	41	None	?Adenylyl cyclase (inhibition)
G_q family	41–43		
$G_{\alpha q}$		None	Phospholipase C (activation)
$G_{\alpha 11}$			Unknown
$G_{\alpha 14}$			
$G_{\alpha 15}$			
$G_{\alpha 16}$			
G_{12} family	44	None	Unknown
$G_{\alpha 12}$			
$G_{\alpha 13}$			

Table 1 Heterotrimeric G-proteins alpha subunits in brain. Taken from Molecular Neuropharmacology. A foundation for Clinical Neuroscience. E J Nestler 2001.

Opiate drugs exert their effects by binding to one of the three opiate receptor types (μ, δ, κ) and mimic the actions of endogenous opiate peptides, the endorphins. However, it is the Mu opioid receptors (MOR) that are critical for the rewarding effects of morphine [Contet et al., 2004]. Not surprisingly MORs are found at a relatively high density in the nucleus accumbens (NAc), the main reward centre of the brain. MOR are also found at high level in the major effluent projections to the NAc including, ventral tagmented area (VTA) and

locus coeruleus (LC) [Walker et al., 2000]. Opioid receptors mediate many of their cellular effects via coupling to, and activation of heterotrimeric G-proteins [Connor et al., 1999] as will be discussed below. Morphine binds to MOR in the LC and VTA and increase the levels of Dopamine (DA) released from the NAc by inhibition of the gamma-aminobutyric acid (GABA)-ergic inhibitory interneurons in the VTA, which in turn disinhibit DA neurons [Taco et al., 2002, Xi 1999]. Increased firing of the DA neurons enhances the rewarding effects of these drugs and induces reinforcement [Taco et al., 2002].

Dopamine (DA) binds two receptors, D1 and D2. D1 receptors are coupled to Gs protein, and have diverse downstream effectors such as increased adenylyl cyclase, and therefore cAMP activity, along with cAMP-dependent protein kinase (PKA) activity. D2-like receptors are coupled to Gi proteins and are negatively coupled to cAMP (see table 1). The binding of DA to each receptor stimulates changes in phosphorylation of target substrates such as glutamate and GABA receptors, as well as ion channels and neurotransmitter receptors. PP1 is a phosphatase that antagonizes many of the actions of PKA, and is negatively regulated by the dopamine and cAMP regulated phosphoprotein (DARPP-32). Therefore the regulation of PP1 proteins is particularly relevant to dopamine modulation (see fig 1.3 from Greengard review). For a detailed description of the multiple aspects of this pathway refer to the review by P.Greengard [Greengard et al,. 1999]. Simply, dysregulation of these systems and long term adaptations are thought to result in the drug addicted state.

1.4 Spinophilin and Protein Phosphatase 1 (PP1)

PP1 is ubiquitously expressed serine/threonine phosphatase that is enriched in the dendritic spines. Dendritic spines are protrusions from the axon that receive about 90% of excitatory synaptic contacts [Harris and Kater, 1994] and are functional elements involved in learning and memory (see front cover-Dendritic Spines). PP1 has been shown to regulate Ca^{2+} currents, agonist induced K^{+} currents and is implicated in the induction of long term depression (LTD) in the hippocampus [Watanabe et al., 2001] and therefore long term

changes or plasticity associated with opiate addiction. PP1 has a broad range of substrates that dictate its subcellular localization, termed targeting subunits, and in some instances its catalytic activity. SPL was found to target PP1 to its substrate and to facilitate the ability of PP1 to dephosphorylate its target. [Yan et al., 1999].

Competitive Binding studies revealed that the residues 438 though 461 of spinophilin bind to PP1 [Yan et al., 1999]. SPL and neurabin are characteristic of cytoskeleton scaffolding proteins (see below) and it is through this property they serve to recruit PP1 to their substrates at the synapse.

1.5 Expression Pattern of Spinophilin

Western blot analysis of brain extracts revealed highest levels of spinophilin protein in the hippocampus as well as striatum, thalamus, hypothalamus. Lower levels of expression were found in the cortex, cerebellum and the brainstem. Light microscopic photomicrography of a coronal section at the level of the hippocampus and thalamus showed immunoreactivity is most intense at the hippocampus. A closer look revealed that this reactivity was concentrated mostly in the dendritic spines head, and it was for this reason that the discoverers of the new protein named it spinophilin [Allen et al., 1997].

1.6 Sequence Analysis and Structure of Spinophilin

SPL is a ubiquitously expressed protein with a molecular weight of about 140 KDa, consisting of 817 amino acids (See fig 1.3-Schematic diagram of endogenous SPL). Database searches revealed a region in the carboxyl terminus of SPL that was similar to many filamentous myosin-like proteins and by algorithm analysis, it was predicted to have a solvent-exposed left handed coiled-coil domain [Allen et al., 1997]. It was proposed that this domain may assist in homologous or heterologous dimerization and may contribute to the scaffolding function of SPL in the cytomatrix of the dendritic spines. The amino terminus of SPL is proline rich and has three Src homology 3-(SH3)

consensus motifs. This domain has recently been shown to bind to actin [Hsieh-Wilson et al., 2003]. SPL also contains a PDZ domain, suggesting that it binds transmembrane protein(s) located in the dendritic spines, thus bringing PP1 into direct contact with these structures. [Allen et al., 1997]. However, more recent data has shown that p70 ribosomal S6 kinase binds to the PDZ domain of neurabin and SPL [Burnette et al., 1998] and may be involved in the regulation of local protein synthesis in dendritic spines and, therefore, plasticity.

Figure 1.3 Schematic diagram of the domain structure of endogenous spinophilin **(Taken from Brady et al., 2003)**

1.7 Phenotypic Characterization of Spinophilin KO Mice

Spinophilin knockout mice have reduced brain size, particularly in the hippocampus and reduced number of layers, possibly as a result of reduced association between P70 S kinase and actin. However, the mice had increased number of dendritic spines, assessed by golgi staining, indicating the possible suppressive role of spinophilin in dendrite formation. SPL KO mice were shown to have a more persistent glutamate receptor currents, no run down time, suggesting a role of spinophilin in targeting PP1 to AMPA, and NMDA receptors for dephosphorylation and subsequent down regulation. Mice had reduced long term depression (LTD) but normal LTP, indicating the targeting of spinophilin/PP1 to the machinery mediating induction of LTD. [Feng et al., 2000].

Behavioral studies with SPL KO mice revealed that mice become tolerant to morphine after a single drug injection. They also showed an intensified opiate withdrawal pattern and the mice appear to be more sensitive to the rewarding effects of morphine as illustrated by the place preference paradigm. Interestingly, mice were also more sensitive to relieving of withdrawal effects by administration of the α2-AR agonist clonidine [Zachariou et al, 2005 submitted]

1.8 Spinophilin and Kinases.

SPL has been demonstrated to interact with a variety of protein kinases, including PKA and PKC, Calcium/calmodulin-dependent protein kinase II (CaMKII) all of which are implicated in the regulation of synaptic plasticity. Also, interactions with P70 s6 kinase and SPL have been documented (Burnett et al. 1998). $p70^{s6k}$ is a member of the protein kinase C family of serine/threonine kinases. This kinase plays a crucial role in the control of mRNA translation by phosporylating the S6 protein of the 40s ribosomal subunit in response to mitogenic stimuli. SPL interaction with kinases is mediated through the PDZ domain of SPL via the C-terminal amino acids of the kinases. These interactions provide evidence that SPL may provide a link between upstream events that regulate mitogen activated gene transcriptions.

1.9 Neurabin and Spinophilin

Neurabin 1 (Neuronal actin binding protein) shares about 48% homology with SPL. The PKA binding sites ser-94 and ser 117 are not conserved in neurabin [Hsieh-Wilson et al., 2003], and differ from those on SPL, indicating that the functional activities between the homologues may be regulated through deferential protein phosphorylation. Neurabin binds PP1 and some kinases including $p70^{s6k}$. Nevertheless a major structural difference between SPL and neurabin is that the latter does not posses a receptor binding domain (RBD) and, as such, does not associated with transmembrane receptors. This suggest that the two homologues have distinct roles in vivo.

Neurabin 1 was first discovered as a binding protein of 70kDa S6 Kinase ($p70^{s6k}$) in 1998 by performing yeast 2 hybrid analysis [Burnett et al., 1998]. In situ hibridization studies revealed a co-localization of the two transcripts with highest expression of both in the granular cells of the cerebellum and in the hippocampus. Neurabin mRNA was also enriched in the striatum, thalamus and the olfactory bulb.

An independent group identified neurabin as an F-actin binding protein with this interaction occurring at the N terminus [Nakanishi et al., 1997]. This interaction targets neurabin to the cytoskeleton compartment. Thus a model of the action of neurabin was proposed by Burnette et al [Burnette et al., 1998] In this model neurabin dimerizes via the coiled-coil domain at the carboxyl end and binds p70s6k in the cytosol of nerve cell body. Subsequently, neurabin binds to F-actin via its amino end and targets the complex to nerve terminals. The role at the synapses of p70s6k is unknown but neurabin appears to augment its kinase activity and it is possible that the kinase is required to modulate translation in response to synaptic demands. Interestingly SPL binds neurabin and possibly modulates its homologues activity.

1.10 Spinophilin and Actin Filaments

As discussed previously SPL has been show to be enriched in dentritic spines. Dentritic spines (see front cover picture) are highly motile specialised protrusions from the axons that receive the vast majority of excitatory input in the CNS. [Harris 1999]. The mechanisms by which the spines change shape in response to different stimuli have been attributed to the dense network of proteins of the actin cytoskeleton. SPL was shown to bind actin by two independent research groups in 1997 [Allen et al., 1997, Satoh et al., 1998] and the results suggests that SPL plays a modulatory role in the changes in spine structure and function. There are several lines of *in vitro* and *in vivo* evidence for this prediction. Firstly SPL has been shown to cross link actin filaments *in vitro* [Satoh 1998] Secondly, as mentioned before, SPL null mice

have reduced hippocampus size and increased number of dendrites during development [Feng et al., 2000]. Thirdly, cultured neurones from SPL knockout mice had increased spine-like protrusions. These results suggest that SPL modulates actin cytoskeleton, either through facilitation of spine retraction or suppression of initial outgrowth of spines from dendrites. SPL, in addition to actin, binds a range of proteins, including its sister, neurabin, arrestins, PP1, kinases and a variety of transmembrane receptors. Through the complexity of these interactions SPL is thought to function as a scaffolding protein, regulating the cross-talk between various physiological stimuli in dendritic spines. It has been proposed recently that the phosphorylation of SPL modulates its interaction with actin. SPL was found to contain 9 phosphorylation serine consensus sites for PKA in the actin-binding domain at the amino terminus between amino acids 1-221 and more over, the phosphorylated state of SPL determined its subcelluar location with dendritic spines [Hsieh-Wilson, 2003]. Due to the observation that SPL is phosphorylated in the actin binding domain the authors went ahead to examine whether this may effect the binding of SPL to actin by performing stoichiometry and a radio-labelled actin overlay assay. It was found that phosphorylation of SPL by PKA directly disrupts its association with F actin, but does not perturb SPLs association with PP1. Therefore, the regulated interaction of SPL with the cytoskeleton may play a role in PP1 translocation.

1.11 Spinophilin and Receptor Interactions

SPL has been reported to bind to a wide range of G-protein coupled receptors including D2 Dopamine receptor [Smith et al., 1999] all three classes of the a2-Adrenergic receptors (AR) [Brady et al., 2003, Richman et al., 2001] and glutamate regulated receptors [Yan et al., 1999]. These interactions are mediated through the receptor binding/interacting domain (RBD) amino acids 169-255 of SPL (see fig 1.3) and the third intracellular (3i) loop of the specific receptor [Richman et al., 2001]. This interaction is specific to SPL as neurabin does not posses a RBD and therefore does not bind to receptors

From the literature, SPL appears to have a number of roles. Firstly SPL was shown to have a tethering or stabilizing role on the α_{2B}-AR at the basal lateral surface of polarized kidney cells. [Brady et al., 2003]. α_{2B}-AR are expressed in the autonomic NS and upon agonist binding act to suppress neurotransmitter release via their link to Gi /Go proteins [Limbird et al., 1988] and decrease cAMP signalling activity. This stabilizing role of SPL on the receptors was observed *in vivo* after agonist induced internalization of transfected tagged α_{2B}-AR, measured by cell surface ELISA, appeared enhanced in SPL absent MEF cells. This implicates the important role of SPL in modulating receptor turn over and signal transduction through the receptor to which it is bound. Two years later Brady et al. demonstrated with HEK293 cells transfected with α_{2B}-AR subtypes and GFP-tagged SPL that there was a 30% increase in SPL translocation to the membrane after agonist compared with baseline [Brady et al., 2000]. Previous studies had shown that SPL is constitutively associated with, or just below, the surface membrane [Satoh et al., 1998, Richman et al., 2001] Brady's team went on to identify the role of G βγ subunit of the G protein in the redistribution of SPL and decided that it was a consequence of signal transduction.

Secondly, in 1999, Smith and collogues, conducted a yeast 2 hybrid screen for binding partners of the Dopamine 2 receptor (DA2) at the third intracellular loop and identified SPL [Smith et al., 1999]. They went on to show this interaction *in vivo* by showing co-localisation of SPL and D2 in cultured epithelial cells. They identified the binding site on SPL as being distinct from that for PP1 and actin and showed that SPL can bind the two proteins simultaneously [Smith et al., 1999].

Both D1 and D2 DA receptors can be found in the dendritic spines of neostriatum, cortex and hippocampus, thus the targeting of PP1 by SPL may be particularly important with regard to the DA modulation of post synaptic glutamate excitatory responses following LTP and LTD. It seems SPL is an intricate member of the D2-like receptor (and possibly the D1) signaling

complex by linking the receptors to down stream signaling molecules and the actin cyto-skeleton.

Another study identified SPL's role in the regulation of AMPA-type glutamate receptor channels through the D1 receptor/cAMP/PKA cascade and therefore SPl has been implicated in regulation of long term depression (LTD), and synaptic plasticity. In the model proposed by Yan and colleagues, in the absents of dopamine(DA), SPL targets PP1 in the vicinity of the AMPA channel and maintains the channel in a dephosphorylated 'low activity' state, which favors Na^{2+} influx. On the other hand, when DA binds DR1, this mediates cAMP activation of PKA and DARPP-32 and thus inhibits PP1 binding to the channel. This synergistically increases phosphorylation of AMPA channels, preventing Na^{2+} influx and thus prevents 'rundown time' of the channel. SPL involvement in this model comes from two pieces of evidence. Medium spiny neurons were taken from spinophilin KO mice and rundown time measured by whole-cell punch clamp; it was found to be greatly reduced [Feng et al., 2000]. In addition, disruption of the spinophilin/PP1 targeting complex also led to modulation of channel current. A peptide of SPL (438-461aa) that contained the docking motif for PP1 was synthesized and this antagonized the PP1/ full length SPL interaction. When this peptide was infused into medium spiny neurons, the run down time of AMPA channels was also prevented. This effect is not attributed to PP1 inhibition but to the disruption of PP1 targeting by SPL to the channel. This prevents dephosphorylation and associated loss of activity [Yan et al., 1999].

1.12 Spinophilin is a Functional Antagonist of β-arrestin

Arrestins (β Arr) are essential modulators of GPCR activity and signaling. β Arr was initially found to have only a desensitizing role on GPCR, hence the name. After agonist binding, it is proposed that G protein-coupled receptor Kinase (GRK) phosphorylates the specific GPCR, facilitating the sequestering of β Arr, which further uncouples the receptor from the G protein, finally leading to desensitization of the receptor. β Arr has also been shown to have a role in targeting the receptor to endocytotic machinery thus playing an

essential role in receptor internalization [Attramadal H et al., 1992, Bohn LM et al., 2000]. Recently, work with βArr null mice [Wang Q et al., 2004] has shed new light on the dual role of arrestins as players in signal transduction. They showed arrestins can also promote signaling by linking GPCR to Erk and MAPK signaling pathways. SPL has now been show to be an antagonist of βarr2 by competing for GRK2 association on the GPCR. By doing so, SPL attenuates βarr2 function and causes dephosphorylation of the receptor, prevents arresting dependent signaling and βarr2 mediated endocytosis of AR receptor. However, it is important to note that the complex inter-relationship involved in GPCR desensitization and internalization differs depending on the type of agonist, GPCR, and the cell milieu in which it is studied [Ferguson et al., 2001]. What is clear is that βarr2 contributes to the regulation of each of these processes, and SPL antagonizes βarr2.

1.13 Spinophilin and Regulators of G protein Signaling (RGS)

Regulators of G-protein Signalling (RGS) proteins are a growing class of proteins that activate the G Proteins α subunit's intrinsic GTPase activity. In turn this reduces the duration of GTP-bound activated state of the subunits and returns them to the 'low' state, inhibiting G protein function [Gold et al., 1997]. To date, thirty RGS proteins have been identified in mammalian tissues. RGS proteins are typified by the highly conserved RGS domain, (which is also shared in Arrestin family of proteins) that conveys the GAP activity. This RGS domain is flanked by the C and N terminal domains. *In vivo* the RGS proteins act in a GPCR specific manner, with the N terminal participating in recognition of the GPCR 3i loop [Wang X et al., 2005]. RGS proteins have regional specific expression pattern in the brain, demonstrating that RGS proteins play an important role in determining the intensity and specificity of neuronal signalling pathways [Zachariou et al., 2003]. Wang Q. proposed in 2004 that SPL passively regulates GPCR signaling by displacing βarr2. However, a year later collaborators further analyzed the role of SPL on AR signalling and discovered that SPL actively regulates signaling by recruiting several RGS proteins to the vicinity of the agonist-AR-βγ complex.

Furthermore, they showed that binding of SPL to the 3i loop of the receptor was absolutely crucial for RGS mediated inhibition of a2-AR Ca^{2+} signaling [Wang X et al., 2005). Taken together, the results provide a molecular mechanism that explains how the binding of SPL and the displacement of βarr2 attenuates signaling by GPCR. It is possible that the interactions between the array of RGS proteins, Gα subunit types and GPCR are regulated by SPL to convey signaling specificity.

1.14 Aims of the Project

To date the *in vivo* regulation of SPL by morphine has not been documented. Therefore in order to investigate if SPL plays a role in opiate signaling, the present study examined, by western blot, the levels of SPL protein in the Nac of WT mice exposed to either acute or chronic doses of morphine.

Behavioral experiments have revealed that morphine addiction is heightened in SPL null mice thereby implicating a role for SPL in the regulation of the addictive state. To explore this observation further two distinct cell culture models were used to observe the subcellular localization of MOR and SPL in morphine treated and untreated cells and to identify if the two proteins co-localize.

2. MATERIALS AND METHODS

Unless otherwise stated, all materials used during the course of this study were obtained from Sigma Ltd and the product code is given.

2.1 Preparation of Competent Cells

Bacteria cells (DH5B) were grown overnight in 5ml medium 2XTY (see appendix A) on a shaker at 37˚C. 2.5ml of this culture was added to 100ml of fresh medium and grown for an additional 2 hours until mid log phase (O.D. 600=0.7). At this point the flask was put on ice for 10mins. Cells are were then placed in chilled falcons and centrifuged at 3900rpm for 15mins at 4°C. Pellet were gently resuspended in 500µl of pre-chilled 2XTY medium and then 2.5ml of LB-PEG medium is added and the cells were aliquoted out and stored at -80°C for up to three months.

2.2 Bacterial Transformation and bacterial Preparations

Competent bacterial DH5B cells were thawed on ice and 100µl were immediately mixed with 5-10ng of desired plasmid DNA. Cells were left on ice for 15 minutes and then heat-shocked for 1 minute at 42°C. They were then returned to ice for 5 minutes. 900µl of 2XTY medium was added aseptically, and incubated for 1 hour at 37°C on a shaker. 100µl of cells were then plated out on plates containing 2XTY medium together with agar and ampicilin (see appendix A) and left over night at 37°C.

Colonies were picked from the plates and standard mini-scale preparation of plasmid DNA was carried out to confirm that the bacteria had successfully taken up the spinophilin construct. The products were visualized via agarose

gel electrophoresis and confirmed by digestions with the restriction enzymes Hind III and Xho I. These two restriction sites are within the polylinker and not within the spinophilin gene and give two expected products of length 4300bp and 5446bp. Once confirmed a medium scale preparation of the colony was performed with QIAGEN kit (Cat no 12243), according to the protocol from Qiagen (See protocol from Qiagen midi and maxi prep kits) and the presence of the bacteria transformation was again confirmed by digestions.

2.3 Cell Culturing Methods

2.3.1 General Procedures- HEK-293

The Human Embryonic Kidney Cells (HEK293) were maintained in continuous culture in 75cm^2 flasks (Cornings) incubated at 37ºC, 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) containing 2% Penicillin/streptomycin (Pen/Strep) , L-Glutamine (Gibco) and 10% fetal calf serum (Gibco). At 80-90% confluency the cells were split using Trypsin (Gibco) and either reseeded into new sterile flasks or used for experiments.

2.3.2 General Procedures-PC12 Cells

PC12 cells are a cell line derived from a pheochromocytoma of the rat adrenal medulla and are valuable for studying neuronal cell models. PC12 cells have endogenous MOR and downstream signalling machinery and due to this feature single transfection experiments could be carried out. Cells were maintained in the same conditions with HEK293, except that the DMEM was supplemented with 10 % FBS, 1% Pen/Strep.

2.3.3 Transient Transfections

Transfection for both cell types was carried out in 6 well plates using lipofectamin 2000 (Invitrogen) following the recommended protocol. Cells were first counted using a haemocytometer and plated out at 5x10^5 cells per well.

Before transfection the plasmids were first re-precipitated from the midi-preparation with 2 times the volume of 70% ETOH and 1/10 the total volume of Sodium Azide (S-8032), left at -80°C for 20 minutes and then spun at 4°C for 20 minutes at 14,000 rpm. This was done in order to re-suspend the plasmid in sterile H_20 for cell culture use. Plasmid DNA was then quantified by spectrophotometry and 2µg of plasmid DNA and 4µl-6 µl of Lipofecatimine was generally used. The following plasmids where used for the range of experiments: HA-tagged MOR plasmid in pCDNA3.1, Spinophilin in plasmid vector pCDNA3, GFP-spino in pEx39GFP and the corresponding vector alone for control experiments (see appendix B for Plasmid maps). The cells were cultured in DMEM medium enriched with 10% FBS one day prior to transfection, and during transfection OptiMEM medium (Gibco) with serum free conditions were used in order to increase transfection efficiency. Cells were left for 18 hours and then 1ml of 20% FBS was added to make a final concentration of 10% FBS. The cells were left to recover for 4 hours before being treated with saline, morphine (100µM) or DAMGO (5µM) for the specified treatment time. PC12 cells, which did not need immunocytochemisrty, were first washed before being mounted on slides with 10% mowiol (Calbiochem, 475904) and viewed by confocal microscopy at oil objective lens 40X. All experiments, unless otherwise stated, were repeated in triplicate.

2.3.4 Immunocytochemistry in HEK293 Cells

Sterilized cover slips were placed in the wells of a 6 well plate. Poly-L-Lycine solution (P 8920) was diluted 1:10 in sterile water and then filter sterilized in the hood before use. 600µl was added to each coverslip and the plate was left for 20mins in the incubator. The liquid was then aspirated and the plates left to dry for at least 20 minutes in the hood before cells were added.

HEK293 cells were left for 24 hours in 10%FBS DMEM before transfection and a further 24 hours before treatment. Treatment of cells was with 100µM morphine, 5µM DAMGO and saline, which was used as a control. Treatment was carried out for 10mins except for the time course experiment. Cells were then fixed for 20 minutes with 1:10 formaldehyde and blocked in 3% milk,

0.1% Triton X, 1mM Ca Cl_2, 50mM Tris Cl for 20 minutes. The primary antibody for the HA-tagged MOR plasmid was diluted 1:1000 in blocking solution, added to the coverslips and incubated for 45 minutes. Cells were then washed in 1XPBS and blocking solution was added for a second time for 5 minutes before the secondary antibody was applied for 20 minutes in the dark. The secondary antibody used was Cy3 colour-conjugated donkey anti-mouse diluted at 1:500 in blocking solution. Cover slips were then washed and mounted onto slides with 10% moviol and examined with Laser Confocal microscope with a 40X oil-objective lens.

2.4 Animals

Mice were housed in a standard animal facility with 12-h light/dark cycle and free access to water and food. Generally, animals were kept together with 2-4 siblings of the same sex. All animal procedures were approved by the Institutional Animal Care and Use committee and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory animals. For acute morphine administration, an i.p injection of morphine sulphate (NIDA) (15 mg/kg) was carried out and then the animals were decapitated 10 minutes or 2 hours after injections. For chronic morphine administration 25mg morphine pellets (NIDA) were s.c. implanted to the mice for 3 days and then the mice were sacrificed.

2.5 Genomic DNA Preparation.

Mice tails were incubated overnight in 700µl Lysis buffer (see Appendix A-solutions) and 25µl of Proteinase K (20µg/µl) (P-6556) at 55°C. 10µl of RNase (10µg/µl) was then added for 1½ hours at 37°C. Phenol:Chloroform (5:1,acid equilibrated: pH 4.7, P-1944) DNA extraction was then carried out which consisted of adding equal volume of Phenol:Chloroform, shaking vigorously followed by 20 minutes centrifuge at RT, 14,000rpm. After transferring top phase to new tubes, 0.6% of volume of 2-Propanol (I-9516) was added and DNA was removed using flamed glass pipette tips. The DNA was washed in 70% ETOH before being left to dry for 15 minutes. The DNA was then allowed

to dissolve in an appropriate volume of double distilled H_2O overnight at 4°C. The genomic DNA was stored at -20°C until used for PCR reactions.

2.6 Polymerase Chain Reaction (PCR)

PCR was carried out for genotyping of the animals prior to experiments. Below is the standard recipe used for each PCR. The expected wild type band was 550 bp and for knock out it was 330 bp.

Betaine (B-0300)	5µl
Primers (IMBB)	0.5µl
10X Taq polymerase buffer (Minotech)	2.5µl
2mM dNTP (Minotech)	2.5µl
25mM Mg Cl (Minotech)	1.25µl
Taq polymerase (Minotech)	0.5µl
DNA	0.5µl
ddH_20	up to 25µl

The running conditions for spinophilin were as follows:

Step 1 - Initial denaturation	94°C	5 mins
Step 2 - Denaturation	94°C	30 secs
Step 3 - Annealing	62°C	30 secs
Step 4 - Extension	72°C	1 min
Step 5 - Cycles	Go to step 2 (30x)	
Step 6 -Final Extension	72°C	7mins
Step 7-End	4°C	for ever

The PCR products were analyzed using 2% agarose gels.

2.7 Western Blotting

Mice were decapitated and the Nucleus Accumbens was immediately dissected and washed in cooled 1XPBS (Invitrogen) before being frozen on dry ice and stored at -80°C. The gel apparatus (Bio-Rad) and glass plates were cleaned thoroughly before use. The separating gel was then made (see appendix A) and left to polymerise for 45 minutes at which point the stacking gel was prepared and allowed to stand for a minimum of 30 minutes. The samples were defrosted on ice and then sonicated in 1% SDS (L-4509), 1% proteinase inhibitor (P-8340). The protein concentrations of each sample were assessed using a modified method of Lowry et al (Lowry et al., 1951), which permits quantification of SDS containing samples (Bio-Rad). A standard curve of bovine serum albumin diluted in SDS was established after which Biorad solutions were added to standards and samples and the absorbance read on a plate reader set at wave length 750nm. The concentrations of the samples were calculated from the standard curve using an excel spread sheet. Exactly 20µg of protein was run for comparison. The samples were then prepared on ice with the appropriate volume of H_20 and protein and sample buffer with 2-mercaptoethanol (FLUKA biochemika, 63689) was added to each before being boiled for 4 minutes and then loaded. Free wells were filled with appropriately diluted sample buffer and kaleidoscope ladder (Bio-Rad, 161-0324) was added for a marker of size in KD. The gel was then run at 30volts through the stacking gel and then increased to 100 volts for adequate time. Transfer was carried out for 1 hour, at 100volts with pre-chilled transfer buffer (refer to Appendix A) using Bio-rad nitro-cellulose 0.45µm membrane (162-0115). The membrane was then blocked for 1 hour with blocking solution 3% milk PBS-1% Tween-20 (P5927) and incubated with the primary antibody. The anti-rabbit spinophilin antibody (kindly donated from Allen PB) was diluted at 1:10,000 in PBS (Invitrogen) and incubated over night at 4°C on a shaker. Lack of immunostaining in knock out spinophilin mice and previous western blots demonstrated the antibody specificity [Allen PB et al., 1997]. The membrane was then washed in PBS-Tween-20 three times before being incubated with anti-rabbit secondary antibody conjugated to horseradish peroxidase (Pierce, 1858415). The antibody was diluted at 1:7000 in blocking

solution and incubated for 1 hour. The membranes were then washed again in PBS 1% tween solution before immunoreactivity was detected by chemiluminescence using supersignal West Pico chemiluminescent substrate (Pierce). Specific bands were quantified using densitometry and equal loading of protein was assessed by Ponceau (P-7170) staining.

2.8 Immunohistochemistry

Mice brains were taken from 4% paraform-aldehyde (P6148) perfused animals and stored for one day in 30% sucrose at 4°C before being cut into 35µm slices with a crytometer and stored at 4°C in PBS 0.05% Na AZ (S-8032). Slices were then mounted onto slides and left to dry for 4 hours before being washed in PBS and then blocked in 0.3% Triton X, 3% Normal Donkey Serum (Jackson Immuno Research, 017-000-121) for 1hr at RT in the dark. For preliminary dilution studies, with dilutions ranging from 1:5000-20,000 the primary antibody rabbit anti-spinophilin and guinea pig anti-MOR (Chemicon, AB1774) were diluted at of 1:10,000. The antibody was diluted in blocking solution and incubated for 16 hours at 4°C. After the incubation period, the slides were washed three times in PBS before incubation with secondary Cy2 donkey anti-rabbit IgG (Jackson Immunoresearch, 711226-152) or Cy3 donkey anti-guinea pig IgG (Chemicon, AP193C) for 4 hours at RT. A dilution of 1:600 was found to give the strongest signal was. Slides were again washed and left to dry for 1-2 hours before being gradually dehydrated in 70%, 96%,100% ETOH and then immersed in Xylene for 3mins and then fresh xylene for 10mins before being immediately cover slipped with hard set mounting media for fluorescence (Vector, H-1400). The slides were visualized on a confocal microscope with a 40x oil-objective lens.

Free floating immunohistochemistry was also carried out to attempt to increase the specific binding of MOR antibody. The procedure was identical to the above procedure except for a few minor modifications. The brain slices were not mounted on the slides and left to dry, but washed immediately in small mesh baskets and incubated with the antibodies according to previous

conditions. After washing the slices were mounted on slides and left to dry at RT for maximum of one hour, vertically, to allow liquid to run off.

2.9 Design of Spinophilin Primers

Using the program Blast search the full sequence of mouse spinophilin was analyzed to determine the unique enzyme restriction sites of the gene. From this sequence two primers were designed in a region of the receptor binding domain (RBD), region 151-444 aa (see fig 1.3- amino acid structure of spinophilin). The primers were designed in order to splice out the RBD via PCR, creating a truncated mutant spinophilin. In future experiments this could then be transfected into cells to identify the role of the RBD.

The spinophilin primer sequences were as follows:

Forward: AACTCGAAGCTGGTC
Reverse: CATCTGCAGGAACATACTT

3. RESULTS

3.1 Transient Transfection of PC12 Cells.

Due to mounting evidence that spinophilin plays an important role in morphine addiction, PC12 cells, which contain endogenous MOR, were used to visualize the movement of spinophilin after treatment with morphine. After being grown on poly-L-lysine treated cover slips until 90% confluent they were transfected with 2μg of GFP-tagged SPL in serum free conditions. The next day cells were treated with either saline, morphine (100μM) or DAMGO (5μM). DAMGO is a natural derivative of enkephalins which promotes endocytosis of MOR [Li H et. al., 2002]. Treatment was for 10 minutes before cells were fixed in 10% formaldehyde and set on slides with 10% mowiol. Slides were then viewed under the confocal microscope. All experiments were carried out in triplicate (see fig 3.1). In saline treated cells GFP-tagged SPL appeared to be localized close to, or at, the cellular membrane with little SPL in the cytoplasm. However, when the cells were treated with either morphine or DAMGO, SPL appeared to redistribute to the cytoplasm. This could possibly be due to the co-localization of SPL with some other molecule that is internalized on treatment with morphine and DAMGO. MOR is a likely candidate and so further experiments were undertaken to try and address this question.

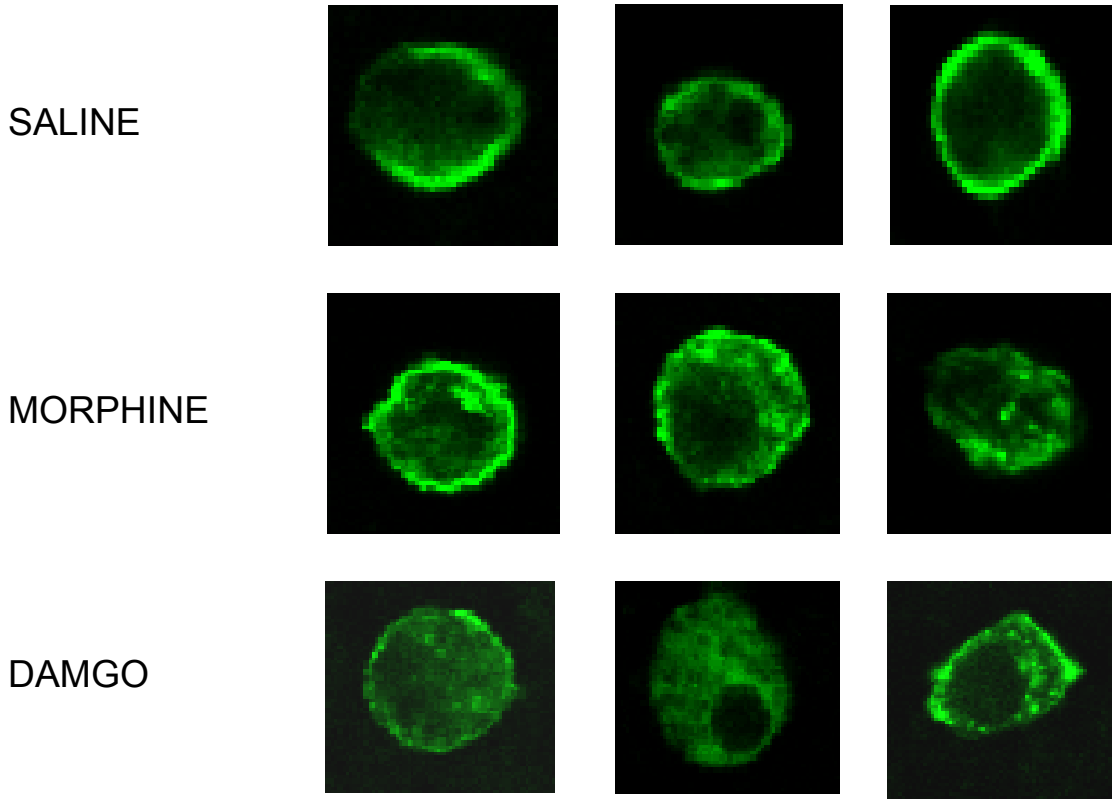

Fig 3.1 GFP-tagged Spinophilin appears to be internalized upon treatment with morphine and DAMGO. PC12 cells were transiently transfected with 2µg GFP-tagged Spinophilin and 24 hours later treatment with saline, morphine (100µM) and DAMGO (5µM). Green fluorescence was observed with confocal microscope using a 40x oil-objective lens. Each experiment was repeated and shown in triplicate. Please refer to section 3.1 for details.

3.2 Transient Transfection of HEK 293 cells

Optimizing conditions were carried out to identify the maximum transfection efficiency dose for SPL (MOR dose had already been established in the lab) and the drug treatment time course for morphine.

3.2.1 Dose Response Studies

Dose response studies with GFP-spinophilin where carried out in order to identify the optimum concentration of the plasmid with the highest transfection rate. HEK 293 cells were plated out according to materials and methods. They were transfected with concentrations of GFP-tagged spino ranging from 1µg-4µg. There appeared an increase in transfection rate at concentrations up to and around 2µg with no increase in efficiency above 2.5 µg. Therefore it was decided that 2µg of DNA would be used for the subsequent experiments. (data not shown)

3.2.2 Time Course for Morphine Response

Once the optimum dose of DNA for transfection had been established a time course was set up to monitor the movement of MOR in the presence and absence of SPL at varying time points of morphine administration. Three time points of morphine administration were chosen; 5 minutes, 10 minutes and 30 minutes. (See fig 3.2). It appears that following 5 minutes of morphine administration, there was a slight internalization of MOR in the presence of SPL compared to control cells transfected with MOR and vector only. Internalization was also apparent at 10 minutes and 30 minutes compared to control cells. However internalization of MOR was more robust after 10min of morphine addition, thus this time point was chosen for subsequent experiments.

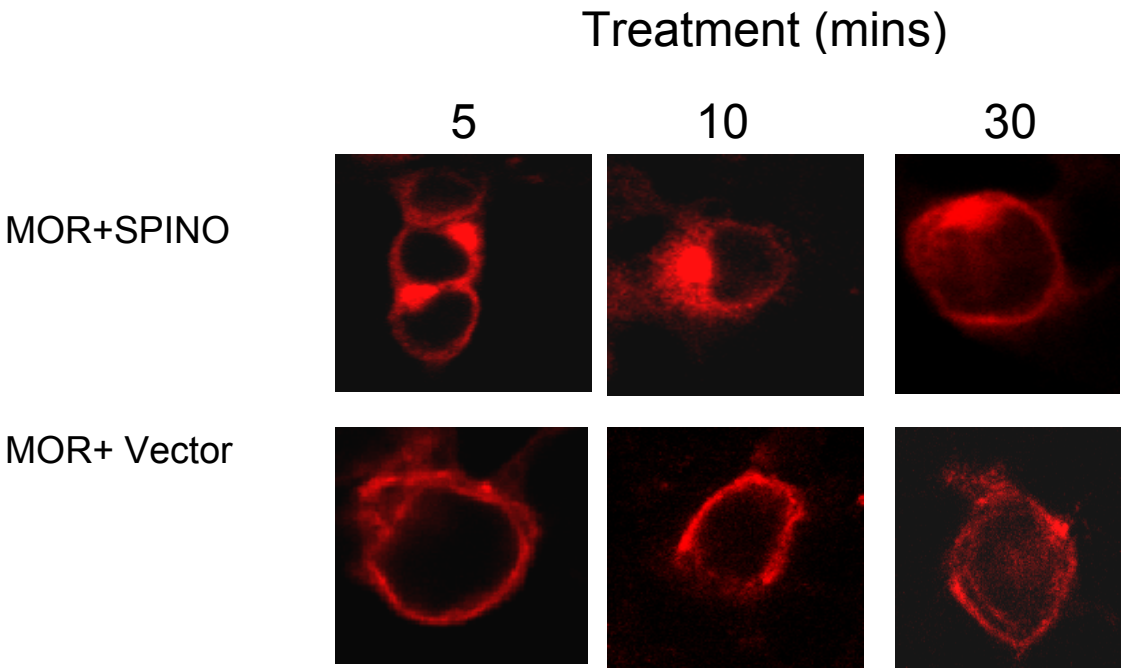

Fig 3.2 Time course for morphine treatment in HEK293 cells transiently transfected with 2µg of spinophilin and 1µg HA tagged MOR or 1µg of HA -tagged MOR and 1µg of control vector (pCDNA3). Cells were grown until 90% confluent on poly-L-lycine treated cover slips before being exposed with morphine for 3 different time points; 5, 10 and 30 minutes. Cells were fixed and the intracellular localization of HA-tagged MOR was determined by immuno fluorescence techniques and observed with confocal microscopy using a 40X oil-objective lens. Refer to results section 3.2.2 for details.

3.2.3 Translocation of MOR to Cell Cytoplasm is Accelerated by SPL

In vivo evidence suggests that SPL knock out mice are more susceptible to generating faster tolerance to morphine [Zachariou V, Allen PB., Charlton JJ SFN abstract 2005, submitted]. The work of He L. provided evidence that endocytosis of MOR can reduce the development of tolerance to morphine [He L. et al., 2002]. Consequently, immunocytochemistry was undertaken to assess whether the presence of SPL affects the trafficking of MOR after morphine treatment in HEK293 cells. After optimizing the conditions, cells were subsequently transfected with 2μg of SPL and 1μg of HA-MOR (see Appendix B- Plasmid maps) or 1 μg HA-MOR and 1μg control vector (pCDNA3) for control experiments. Cells were treated with saline, morphine or DAMGO for 10 minutes and the intracellular localization of HA-tagged MOR was then examined using confocal laser microscopy. As expected, MOR appeared at, or in close proximity to, the cytoplasmic membrane in cells treated with saline in both the presence and the absence of SPL (Fig 3.3, and 3.4, First row). Remarkably, in the presence of SPL, MOR was robustly internalized after treatment with morphine (Fig 3.2 second row), and this occurred within 5 minutes (see fig 3.2). In comparison, cells transfected with MOR and control vector showed no trafficking of MOR upon morphine treatment (Fig. 3.4- Second row). He L. previously documented that DAMGO facilitates a rapid (minutes) endocytosis of MOR [He L et al., 2003] and so was used as our positive control. In both the presence and absence of SPL, DAMGO resulted in the translocation of MOR to the cell cytoplasm (Fig. 3.3 and 3.4 Third row). This result suggests that there are other proteins and possibly other mechanisms working in MOR trafficking besides SPL.

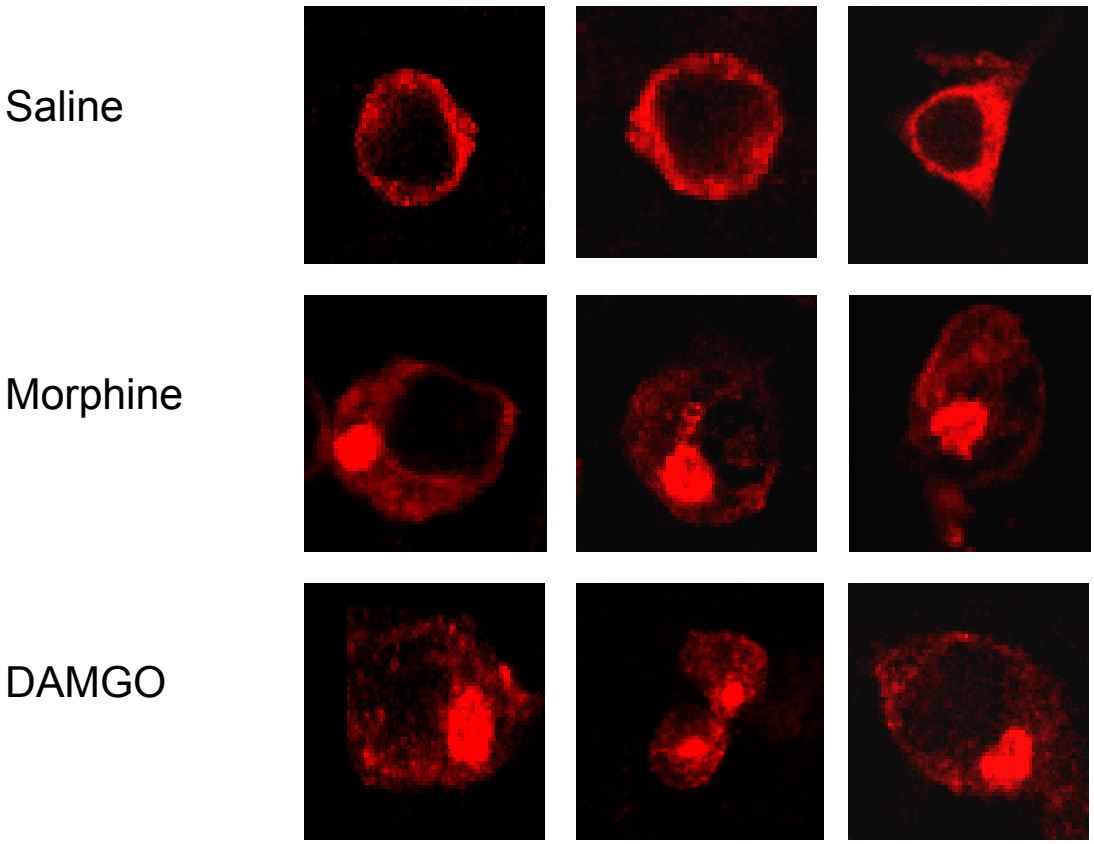

Figure 3.3 SPL accelerates the rate of internalization of MOR with morphine treatment. HEK293 cells were transiently transfected with 1 μg of MOR and 2μg of spinophilin and 24 hours later treatment with saline, morphine (100μM) or DAMGO (5μM). Immunocytochemistry was carried out and the translocation of HA-tagged MOR, seen here in red, was then observed on a confocal microscope with a 40x lens. All experiments were carried out and shown in triplicate. Refer to results section 3.2.3 for details.

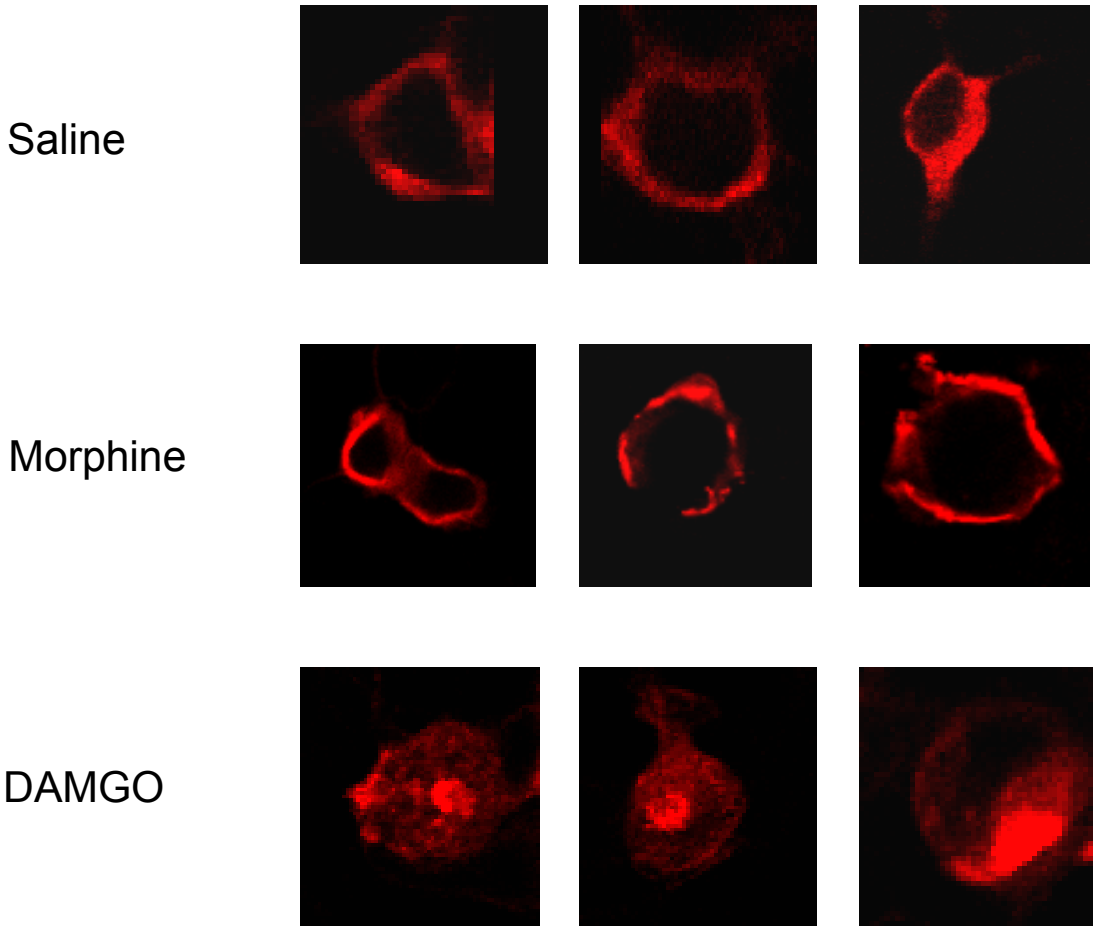

Figure 3.4 MOR does not translocate to the cytosol with morphine treatment in the absence of spinophilin. HEK293 cells transiently transfected with 1µg of HA-tagged MOR and 1µg of control Vector (pCDNA3) and 24 hours later were treated with saline, morphine (100µM) or DAMGO (5µM). Immunocytochemistry was performed to observe HA-tagged MOR distribution by confocal microscopy, shown here in red. Each experiment was performed in triplicate and is shown above. Please refer to results section 3.2.3 for details.

3.2.4 SPL and MOR do not Appear to Co-localize, After Treatment.

Recent evidence has shown that SPL associates with a growing number of GPCR via the 3i loop including AR and DA receptors. It has yet to be determined if SPL directly associates with MOR. Previous experiments with PC12 cells presented here, suggests there is an internalization of SPL after agonist administration. Therefore, it was of interest to discover if SPL is being internalized together with MOR on treatment with agonists. This was achieved by transiently transfecting HEK293 cells with 2µg of GFP-tagged SPL and 1µg of HA-tagged MOR and observing their localization by direct (GFP), or indirect (HA) florescence. Upon treatment with saline, both SPL and MOR were co-localized at the membrane as indicated by the observed areas of yellow on the overlay (fig 3.5 first row). However, after treatment with morphine, MOR was internalized as seen before (fig. 3.5 second row), while SPL appeared to be present at the membrane and cytosol, with only a small area of co-localization show in yellow. The same was apparent after DAMGO treatment; MOR and SPL were internalized however, their trafficking seemed to be independent of each other.

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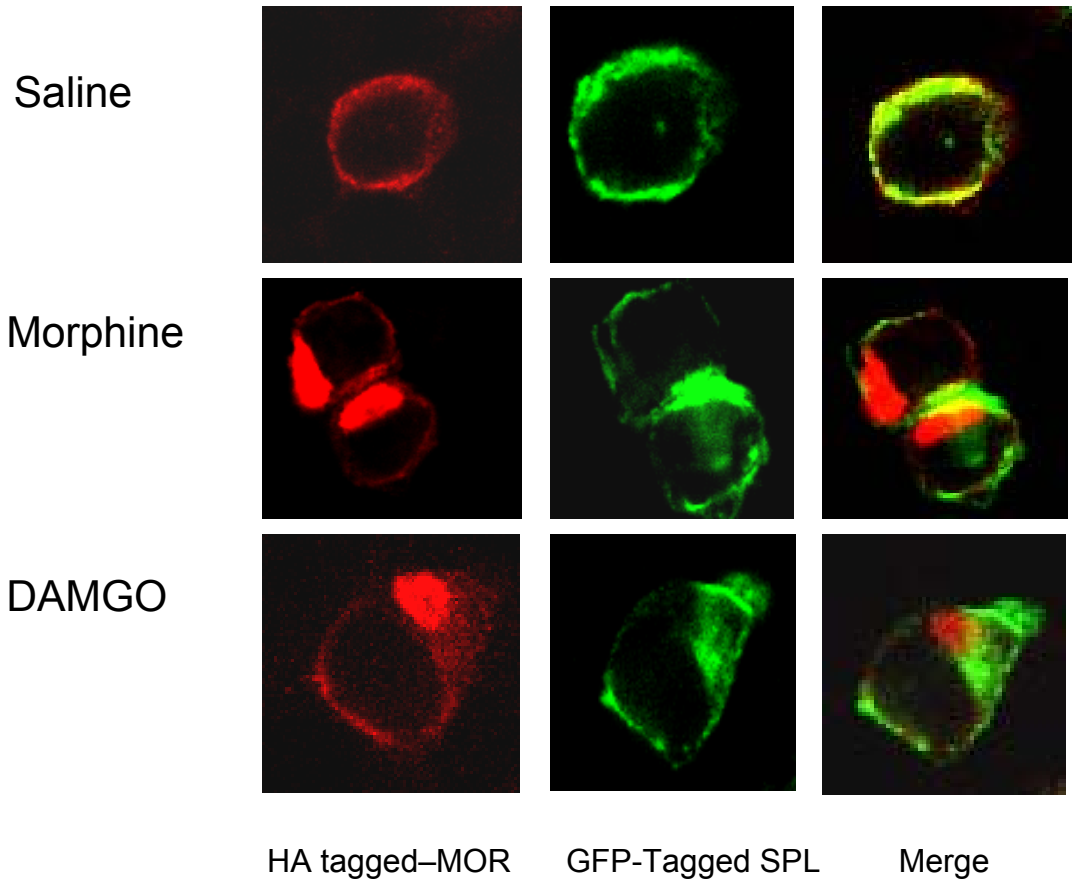


Figure 3.5- Spinophilin and MOR do not appear to co-localize upon drug treatment.
HEK293 cells were transiently transfected with 2µg of GFP-tagged Spinophilin (SPL) and 1 µg of HA-tagged MOR plasmids before being treated 24 hours later with saline, morphine (100µM) or DAMGO (5µM). In the first column the red florescence represents the MOR, in the second column, the green fluorescence represents spinophilin and in the third column, the merge, with yellow representing the overlay of the two colours. Please refer to results section 3.2.4 for details.

3.3 Western Blot Analysis of SPL Protein Levels in the NAc

Previous *in vivo* work in the laboratory has demonstrated that SPL plays an important role in morphine addiction in mice [Zachariou V, et al 2005, submitted]. In order to support these findings by biochemical methods SPL protein expression was measured in the NAc by western blot analysis using a rabbit anti-SPL antibody. A single protein band at 140KD was detected. For acute morphine studies C57/BL6 mice where sacrificed at two time points; 10 minutes and 2hours, post injection. Fig 3.6 and 3.7 depicts the level of SPL in the NAc after a single morphine injection at 10 minutes and 2 hours respectively. The amount of immmunoreactivity of SPL appeared to be reduced after treatment with morphine at 10 minutes compared with saline treated control mice, although this value was not significant. This was a result of high variability between individual samples. An unpaired students t-Test revealed that the level of SPL was significantly decreased at 2 hours post morphine injection (0.33 mean OD) compared the saline-injected subjects (0.53 mean OD, $p<0.01$). See fig 3.7.

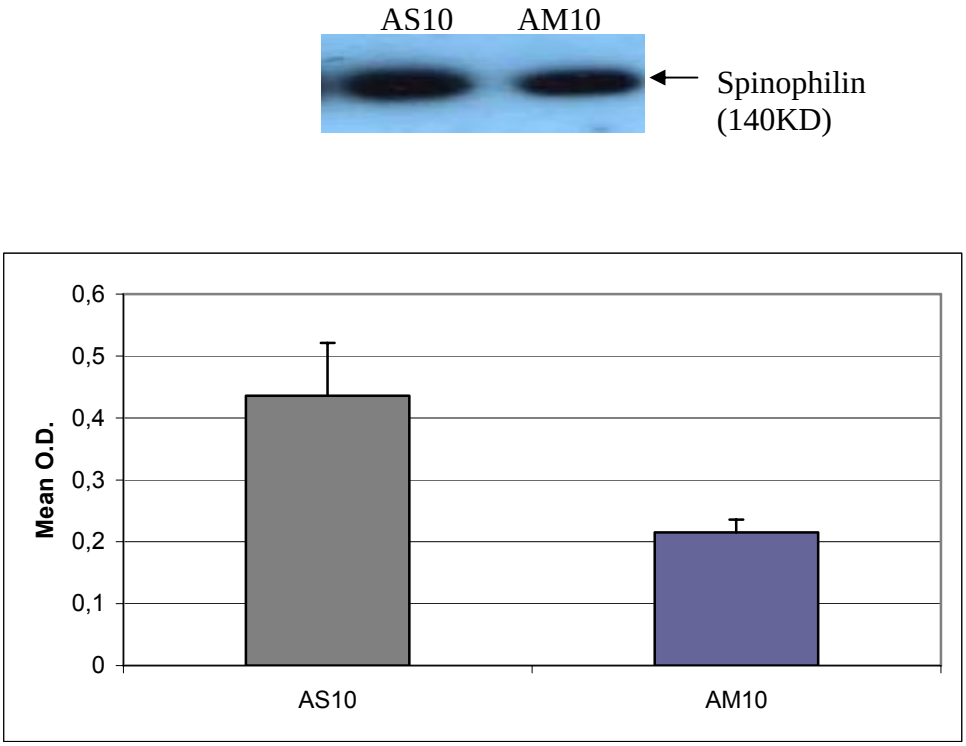


Figure 3.6 Spinophilin levels in the NAc at 10 minutes. Mice were administered a single morphine (AM) (15mg/kg) injection or saline (AS) for controls and sacrificed at 10 minutes post injection. The NAc was dissected immediately and the level of SPL measured by western blot analysis. Shown above the graph is a representative immunoblot of Nac tissue from acute saline and acute morphine injected animals. The band represents spinophilin with a molecular weight of 140 KD. The values in the graph are expressed as the mean optical density (O.D) ± SEM. Refer to section 3.3 for details.

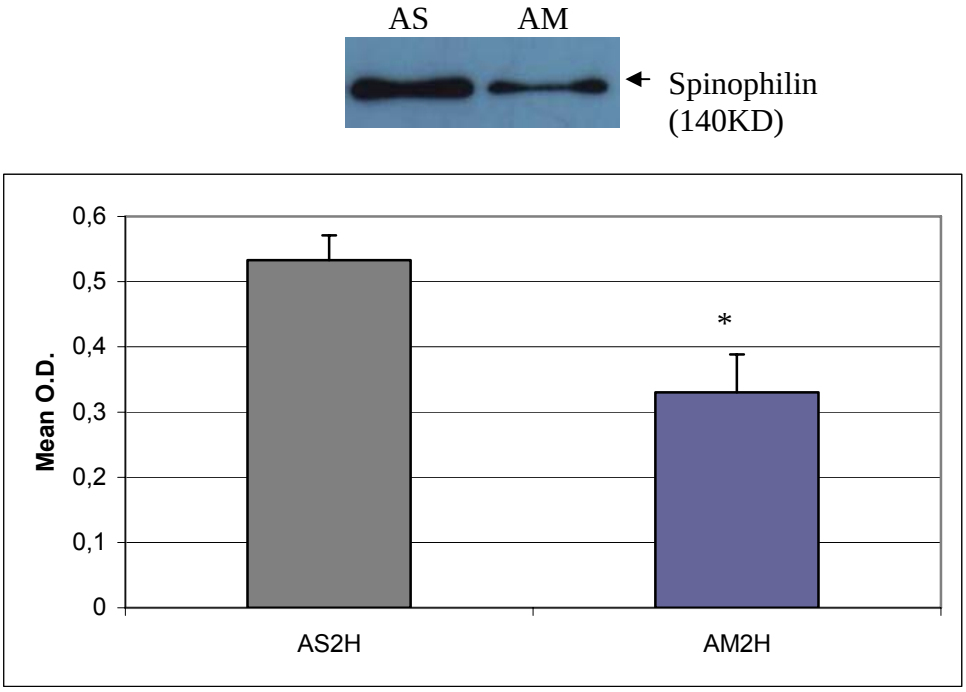


Figure 3.7 Spinophilin levels in the NAc after morphine injection or saline at 2 hours. Mice were administered a single morphine (AM) (15mg/kg) injection or saline (AS) for controls and sacrificed at two hours post injection. The nucleus accumbens (NAc) was then dissected immediately and the level of SPL measured by western blot analysis. Shown above the graph is a representative immunoblot of Nac tissue from acute saline and acute morphine injected animals. The band represents spinophilin with a molecular weight of 140 KD. The values in the graph are expressed as the mean optical density (O.D) ±SEM. * P>0.01. Refer to section 3.3 for details.

In the chronic morphine study, animals had a 25mg morphine pellet s.c implanted to allow for a slow release of morphine for a duration of three days. Fig 3.8 illustrates the SPL immunoreactivity in the NAc after chronic morphine administration. The levels of SPL were significantly increased (0.59 mean OD) compared to mice injected with saline (0.32 mean OD $p<0.005$). These results robustly show that SPL is regulated in response to morphine administration suggesting a role for SPL in modulating some of the extensive molecular and cellular adaptations that occur during morphine addiction.

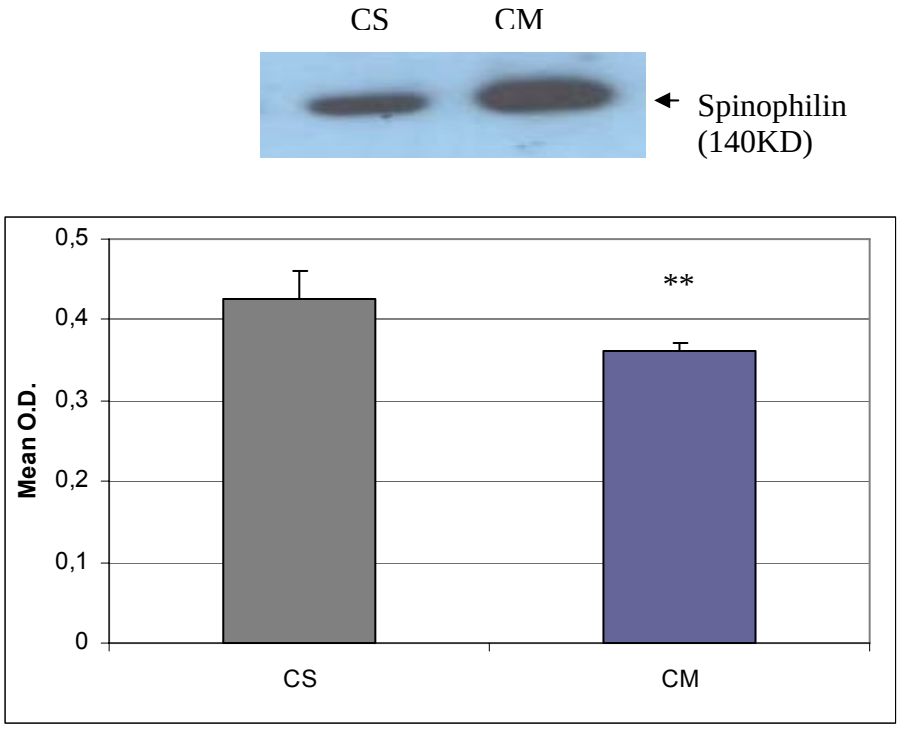


Fig 3.8 Spinophilin immunoreactivity increases in the NAc after treatment with chronic morphine. C57BL6 mice were s.c implanted morphine pellets for a duration of three days. On day three the animals were sacrificed and the Nac immediately dissected and western blot analysis was performed. A representative immunoblot of Nac tissue from chronic saline (CS) or chronic morphine (CM) treated animals is shown above the graph. The band represents spinophilin with a molecular weight of 140 KD. In the graph chronic saline and chronic morphine values are expressed as mean optical density (OD) ± SEM. N=3 **P<0.005. Refer to section 3.3 for details.

3.5 Co-localization of MOR and Spinophilin in the NAc

Unfortunately the results from the SPL and MOR immunohistochemistry experiments were not conclusive, due to the high background staining observed. Through verbal communication and discussion with the collaborating groups it was decided to leave the data out all together.

4. DISCUSSION

The present study revealed four main findings:

- Acute morphine administration in mice resulted in a significant decrease in SPL protein in the NAc, while chronic administration resulted in a significant increase compared with saline treated animals.
- In the presence of SPL, MOR is more readily internalized upon agonist activation.
- SPL, upon morphine administration, appears to be translocated to the cytoplasm.
- SPL and MOR appear to move independently of each other, ie they are not co-localized in the cytoplasm after treatment.

SPL was first discovered in 1997 by two independent research groups [Allen et al., 1997, Satoh et al., 1998] as a binding partner to protein phosphate 1 (PP1). Since then more and more evidence has thrown light on the importance of SPL in the field of neuroscience and beyond. This is a result of a combination of many features of SPL:

- Firstly its location; SPL is densely expressed in the dendritic spines, sites for much excitatory neuro-transmission.
- Secondly, for its multi-domained structure, allowing for the highly fastidious regulation of signal transduction events.
- Thirdly, for SPL choice of partners including actins, phosphatases and kinases, GPCR, RGS proteins, and arrestins.

These attributes place SPL in a pivotal role in mediating signal transduction and the orchestration of the plasticity that occurs in drug addiction.

The present study focused on the role of SPL in morphine addiction. Previous behavioral studies in the lab have highlighted the importance of SPL in morphine addiction. Firstly, mice lacking the SPL gene became immediately

tolerant to morphine after just one exposure. Secondly, SPL knockout mice had a more 'addictive phonotype' revealed by a more intense withdrawal pattern, and thirdly, the mice were more sensitive to the rewarding effects of the drug. These findings prompted the biochemical research needed to support these behavioral observations.

The level of SPL protein, in the nucleus accumbens, was assessed in mice following acute or chronic morphine i.p. injections. Mice that had been exposed to a single morphine dose (15mg/kg) for 10 minutes showed a decrease in SPL protein level (see fig 3.6). However, only at a 2 hours time point was SPL significantly decreased (see fig 3.7). It has been well documented that acute morphine treatment suppresses the expression of many proteins including CREB, pERK [Nestler., 2004, Allen P.B., 2004., Muller et al., 2004, McClung et al., 2005] and therefore due to changes in gene transcription and molecular adaptation, SPL protein level is also reduced. This shows that SPL is a substrate in the adaptive process resulting in the preliminary stages of morphine addiction.

Chronic morphine administration in mice resulted in a significant up regulation of SPL protein in the NAc (refer to fig 3.8). Generally it is accepted that on continual exposure to a drug, homeostatic changes occur to counteract the suppressive effects of the drugs on cellular substrates. This results in negative feedback adaptations that increase gene transcription, most probably via CREB and ΔfosB [Nestler EJ. 2004] and decrease the sensitivity to subsequent drug exposure. Many proteins have been found to be up regulated in many areas of the brain involved in addiction on prolonged morphine exposure. These include Arrestins, GRK, PKA, PP1, GPCR [Nestler EJ., 2004, Allen PB., 2004, Zhang et al., 1998, McClung et al., 2005]. The present study demonstrates that this up-regulation is also true for SPL after chronic morphine administration in mice. Dependence, tolerance and withdrawal symptoms are believed to result from the drug-induced homeostatic adaptation. On cessation of the drug, the overcompensated system is no longer apposed; consequently the adverse effects of the drug appear.

The primary pathway involved in reward is the mesolimbic dopamine neurons (see fig 1.1). Morphine has an indirect effect on this DA pathway by inhibiting the GABA interneurons. This results in an increased activity of the DA neurons from the VTA to the Nac, the reward centre of the brain, contributing to the rewarding effects of the drug. The behavioral studies showed that SPL KO mice were more sensitive to the rewarding effects of morphine analyzed by the place preference paradigm, suggesting that SPL normally plays a role in the dampening of the activity of the DA neurons. This is most likely manifested by regulation of the phosphorylative state of the DA receptor as will be discussed in full below.

MOR are also found in the locus ceruleus (LC). The LC, located in the dorsal pons, is the major noradrenergic (NE) nucleus of the brain and is important for the autonomic nervous system activity [Nestler et al, 2001]. The LC can be implicated in the somatic stress-like effects that result on opiate withdrawal. Initially the activity of LC is inhibited on morphine administration. This is achieved by two means. The uncoupled Gβγ subunit increases the conductance of the K+ channels and causes an inwardly rectifying potassium current. Secondly the Gαi subtype causes the inhibition of expression of adenylate cyclases, the enzymes that leads to the formation of cAMP. The decrease in cAMP secondary messenger results in a decrease in PKA and a subsequent decrease in phosphorylation of target ion channels and pumps. The levels of many other proteins are also affected by the decreased activity of cAMP, including CREB, which results in altered gene transcriptions. Ultimately, this may lead to the long term changes seen in the LC after sustained opiate use. SPL has been shown to be a direct target for the cAMP pathway; PKA has been shown to directly affect the phosphorylated state of SPL [Hseih-Wilson et al., 2003]. This appears to provide a mechanism for regulation of SPL during acute and chronic morphine administration. Following long term opiate exposure the system tries to balance and compensate for the sustained suppression of the noradrenergic (NA) receptor firing in the LC. cAMP levels are increased as are those of many regulatory proteins, including SPL. The activity of the LC is at a new homeostatic level,

which is constantly oppressed by the action of the drug. When the drugs actions are no longer opposing the system, withdrawal symptoms are evident with a far higher firing rate in the LC. In the absence of SPL mice show a more severe pattern of withdrawal. This clearly suggests that SPL is vital for dampening-down the overcompensation of LC activity, by dephosphorylating the receptors responsible for over activity.

The use of morphine for the treatment of chronic pain is hampered by the rapid development of tolerance to the pain relieving effects of the prescribed drug. Initially, it was thought that tolerance was a result of receptor endocytosis, therefore diminishing the number of receptors on the surface that the drug can occupy. The work of He L. and collogues revealed that by facilitating endocytosis of MOR, the development of tolerance is reduced [He L. et al., 2002]. The theory they proposed was contrary to the prevailing theory of the time. The results showed that morphine does not facilitate MOR trafficking giving rise to prolonged receptor signaling ultimately resulting in the adverse effects associated with prolonged drug use. On the other hand, when the receptor is allowed to internalize, arrestin is recruited after phosphorylation of the MOR by GRK, signal transduction through the receptor is ablated, and the receptor is either recycled to the surface for resensitization or follows the path for degradation.

Using a cell culture model the present study revealed that the presence of SPL accelerates the internalization of MOR upon agonist activation (fig 3.3) Consistent with previous reports MOR's were not seen to internalize on the administration of morphine when co-transfected with a control plasmid (fig 3.4).

There is a growing list of GPCR's that SPL has been shown to bind to, including all three types of α2-adrenergic receptor (AR), DA receptors and the two types of glutamate receptors, AMPA and NMDA. However, there is no documented evidence that SPL interacts with MOR making this work all the more interesting. How SPL exerts its influence on MOR trafficking is not clear

but the proposed mechanism by which SPL exerts its regulatory power on drug induced adaptations and receptor trafficking is discussed in full below.

Through studies with knock out mice, β arrestin 2 (βarr2) has been well characterized as a terminator of signal transduction and facilitator of GPCR endocytosis by uncoupling the G protein with the GPCR (reviewed in Ferguson 2001). More recently, data has demonstrated that βarr2 can also have a sensitizing role on specific GPCR and links the receptor to down stream signaling pathways such as MAPK (ERK and JUNK) [Wang et al 2004]. The work by Wang and colleagues on AR revealed that spinophilin binds to the same site, the 3i loop on the GPCR tail, as that of βarr2 and demonstrated that the two proteins appeared to have reciprocal roles *in vitro* and *in vivo*. In α2 AR pathway, where activation of the AR results in sedation as a result of inhibiting the release of adrenalin, βarr2 was shown to participate in the sensitization of the receptor mediated sedation. This highlighted the importance of βarr2 signal promoting property in this particular response pathway. On the other hand, it was shown that in the absence of SPL this sedative response was exaggerated, demonstrating that SPL has an antagonistic role in α2-AR-mediated sedation. Interestingly, these results suggest that SPL cause desensitization of the receptors and curbs prolonged receptor signaling while βarr2 prolongs the AR signaling.

In cell culture work as done by Wang and colleagues, the mechanism by which SPL exerts its regulatory powers was explored. When SPL was over expressed in HEK293 cell it appeared to facilitate dephosphorylation of the α2-AR receptor. They went on to show that this was achieved by competing for GRK2 mediated interaction with agonist-α2-AR-Gβγ complexes [Wang Q et al. 2004]. Additional experiments by the same team revealed that the antagonistic role of SPL on βArr2 went further than originally thought. SPL was shown to have an active role in recruiting RGS proteins to the vicinity of GPCR thus terminating signal transduction and preventing Ca+ currents [Wang X et al. 2005]. In summary, the results proposed by the teams demonstrated that the reciprocal functions of βarr2 in both terminating and promoting GPCR desensitization, trafficking and signaling, are regulated by

SPL. This evidence, in light of the results presented in this study would help to explain the possible mechanism by which SPL regulates the trafficking of MOR and implicates the role of SPL in the prevention of tolerance.

Consistent with previous reports, in this study DAMGO triggered a robust internalization of MOR at 10 minutes. Furthermore, this trafficking of MOR did not appear to be dependent on SPL as both in the presence (fig 3.3 Third row) and absence (fig 3.4 Third row) of SPL, MOR was internalized with DAMGO treatment. DAMGO is a hydrolysis-resistant derivative of enkephalin (a natural endorphin in the body) and is therefore similar in shape and size to the natural agonists of MOR. Owning to this feature, DAMGO is known to readily facilitate internalization of MOR and therefore has a reduced propensity for causing tolerance and dependency in mouse models [Le H. et al., 2002]. Internalization of MOR occurs when bound to a 'good fitted' agonist. Morphine, which is a large molecule and does not 'sit' well in the receptor pocket, causes a delayed internalization of MOR. DAMGO, on the other hand, triggers fast internalization. Previous work by Zhang and colleagues has shown that GRK and βarr2 play a role in the functional modulation of MOR signaling in response to agonist. They showed that over expression of GRK2 alone leads to the internalization of MOR after morphine treatment [Zhang et al., 1998). Furthermore, overexpression of both GRK and β-arr2 in *Xenopus* oocytes results in an attenuation of MOR-activated K conductance (Zhang 1998]. The study indicated that the stability of the receptor activation state required for GRK phosphorylation and βbarr2 binding can be differentially modulated by the binding of distinct agonists. So, it is likely that DAMGO-MOR interactions lead to a conformation that allows GRK-mediated phosphorylation and subsequent internalization [Zhang et al., 1998]. It is interesting that SPL competes with the same site in the 3i loop of agonist occupied GPCR as that of GRK2 [Wang Q. et al., 2000]. It is possible that when SPL is over expressed it competes for GRK action, while in the absence of SPL, GRK is present endogenously in HEK293 cells and is able bind to the specific site, both ultimately resulting in the internalization of MOR on DAMGO administration. The other possibility is that there are endogenous low levels of SPL in HEK cells, and that when the MOR is bound to DAMGO, the

configuration of the R-agonist allows for the endogenous SPL to bind and internalize the MOR. In contrast, the morphine-R conformation is ‘stuck’ on the cell surface and requires high levels of SPL to trigger internalization.

MOR is internalized in the presence of SPL as rapidly as 5 minutes after morphine treatment. Even after 30 minutes of morphine treatment the MOR remains internalized (fig. 3.2). These results demonstrate the importance of SPL in the recycling of the receptor and emphasise the speed and the duration at which SPL assists MOR trafficking. It is possible that upon DAMGO treatment there is a difference in time of internalization in the presence and absence of SPL.

The precise mechanism by which SPL facilitated endocytosis of morphine-activated MOR remains an intriguing question, and one that is being actively pursued. However, the internalization of agonist occupied MOR in the presence of SPL is consistent with the behavioral data; SPL is important for the prevention of tolerance, therefore the results presented above are evidence for the new leading hypothesis that recycling of MOR helps prevent tolerance development and it is clear that SPL plays an active role in this trafficking.

Since internalization of MOR upon agonist treatment, in the presence of SPL, was seen, the next step was to investigate SPL trafficking with and with out MOR agonists. PC12 cells are known to contain endogenous MOR and so were chosen as an adequate cell culture model as they required only single transfection. It was found that in saline treated cells, GFP-tagged SPL resides at the plasma membrane. Interestingly, however, after treatment with both morphine and DAMGO, GFP-tagged SPL appeared to be located mostly in the cytosol (fig 3.1). These results are contrary to the results of Brady and colleagues observed when looking at SPL translocation in HEK cells co-transfected with SPL and AR subtypes. They found that SPL, upon AR agonist treatment, led to the enrichment of SPL at the cell surface within a two minute time period and no change was observed after 30 minutes [Brady et al. 2005]. They also had previously shown that α2B-AR on mouse embryonic

fibroblasts taken from mice lacking SPL, internalized when given agonist, suggesting SPL stabilizes the receptor on the cell surface in this particular response pathway [Brady et al., 2003, Wang et al., 2004].

When taken together, the previous findings in the AR pathway and the results presented here, suggest a differential and possible reciprocal regulation of AR and MOR in relation to SPL. While AR are stabilized on the surface in the presence of SPL, MOR are internalized. Secondly SPL is internalized on morphine administration, meanwhile, upon AR agonist, the protein is enriched at the cell surface. Brady and colleagues demonstrated that this was a result of down stream signaling pathways rather then the affinity of the receptor.

SPL has previously been shown to contain nine consensus sites for phosphorylation by PKA and differentially phosphorylated SPL was found to be localized to specific subcellular compartments [Hsieh-Wilson et al., 2003]. Phosphorylated SPL at ser-94 was enriched in a membrane fraction including postsynaptic densities (PSD). In contrast, SPL phosphorylated at ser-177 was absent from PSD and associated with synaptic plasma membrane fraction and, importantly, in the cytosolic S3 fraction. Thus, the delicate balance of kinase and phosphatase activities in neurons may control the targeting of SPL within dendritic spines [Hsieh-Wilson et al., 2003].

Clonidine, an α2-AR agonist, is used in the treatment of opiate addiction and is administrated to relieve most of the symptoms associated with opiate withdrawal [Raith et al. 2004, Georges et al 2003, Kaye et al. 2003]. These symptoms have been associated with the increase in sympathetic activity in the LC neurons, the major noradrenergic nucleus. This increased activity from the LC was shown to inhibit the firing of the DA neurons. Clonidine effectively relieves this inhibition caused on withdrawal of opiates [Georges et al., 2003]. Interestingly, SPL KO mice were shown to be more sensitive to Clonidine treatment. Experiments from our lab revealed that although spinophilin knockout mice show decreased sensitivity to morphine's pain relieving effects, they are more sensitive to the analgesic effects of clonidine [Zachariou V. et al., SFN abstract 2005, submitted). Supporting the *in vitro* findings presented

earlier, the behavioral data also implicates a differential role of SPL in regulation of the AR and MOR. This different regulation by SPL of the two types of receptors remains to be investigated but it is hypothesized that this regulation is exerted through the various proteins SPL partners with, including the RGS proteins that show a specific brain region distribution (Gold et al 1997., Gold SJ. and Zachariou V., 2004).

The specifics of the variable regulation of SPL location with different GPCR is unclear and remains to be investigated. Nonetheless, these findings taken together in the context of the presented literature indicate that there is indeed a preferential capability of SPL to regulate α2A-AR and MOR and for the receptors to effect redistribution of SPL. The functional relevance *in vitro* and *in vivo* remains to be determined by future studies.

Finally, the extracellular signal-regulated Kinases (ERK)/MAPK pathway is one of many signaling cascades involved in cell regulation, including cell proliferation, differentiation and survival [Mazzuchelli et al., 2002]. Recent evidence has also shown that ERK1 and 2 are important for several forms of learning and more specifically LTP in the hippocampus. ERK1 KO mice have been shown to have an increase in synaptic plasticity accompanied by an increase in sensitivity to the rewarding properties of morphine as assessed by the conditioned place preference paradigm [Mazzuchelli, 2002]. These results provide evidence that neural adaptations, as a result of taking drugs of abuse, share the same molecular elements with those essential for synaptic plasticity. ERK has been shown to be regulated by the cAMP/PKA pathway. After chronic or acute morphine administration, ERK was shown to be regulated in a brain-region specific manner [Muller D et al., 2004]. Acute morphine administration down-regulated pERK levels compared to saline in the NAc but not in the basal ganglia nuclei caudate and putamen. However, after chronic morphine administration, levels where raised in the NAc suggesting a tolerance arises to the inhibitory effects of morphine on ERK activity during chronic morphine treatment [Muller et al., 2004]. In light of this data, preliminary experiments were undertaken to identify if SPL plays a role in this signal transduction pathway. It was found that in KO SPL mice,

morphine fails to decrease pERK levels in the Nac. This exciting new data, further emphasizes the major modulatory role of SPL in the complex process of addiction.

Conclusions

The emerging role of SPL in the adaptive changes that occur during drug taking is clearly demonstrated in the present study. More specifically SPL was shown to be a cellular substrate resulting in the short term and long term changes that occur in morphine addiction. SPL was shown to accelerate the trafficking of MOR, after agonist binding, in the cell culture model, demonstrating a cellular mechanism for the 'addictive' phenotype seen in SPL KO mice. SPL location was also clearly shown to be regulated by morphine; upon MOR agonist, GFP- tagged SPL translocated to the cytoplasm, indicating that SPL is an active player in the MOR signal transduction pathways. Further work is clearly needed to identify the exact modulatory role of SPL in morphine addiction and this is being actively pursued.

Future Work

To ensure that what was seen in this study with cell culture model was not just an artifact of the HEK293 cell model, it would be preferable to grow and then stably transfect neuronal cultured cells. Alternatively, the localization of transfected GFP-tagged SPL in HEK293 cells could be monitored by real time confocal microscopy, using each cell as its own control, before and after treatment. This would enable a more accurate quantification (i.e. % change in membrane fluorescence) in any one cell, before and after treatment. This would essentially increase the accuracy and the power of the presented results.

In the present study we demonstrated that SPL facilitates the endocytosis of MOR after agonist treatment. However it was not shown where in the cytoplasm the MOR resided after treatment. Therefore a future experiment could be undertaken to use co-localization studies with markers for endosomes, or lysosomes etc, in order to identify if the MOR is targeted for recycling, or degradation. Furthermore, a whole array of co-transfection experiments could be done to identify if RGS proteins effect MOR trafficking.

Since SPL has been shown to bind to RGS2, it would be interesting to observe if the level RGS2 alters in SPL null mice. Moreover, since the pain reflex is also housed in the spinal cord, it would be of interest to discover if there is detectable level of SPL protein in the spinal cord. This could be achieved by western blot analysis.

Tolerance to morphine can occur due to the superactivation of the cAMP signaling pathway as a result of maintained signaling through the receptor. An adaptive effect alters the base line MOR expression in cells. RT PCR would be an important control experiment to undertake to show that the behavioral effects seen in the SPL knockout mice are not due to simple changes in MOR receptor density.

It would be also of interest to look at tolerance in a cell culture model by measuring super activity of cAMP after morphine treatment.

To date, SPL has been shown to interact directly with many GPCR including DA2 and AR. From the results presented in this work, SPL appears to be an integral member of the down stream signaling machinery in the MOR pathway. It would be of great value to clearly demonstrate, ideally both *in vivo* and *in vitro*, the direct interaction of SPL with MOR. This could be achieved through a variety of methods including co-immunoprecipitation techniques and yeast two-hybrid assay.

APPENDIX A

SOLUTIONS

1) Media for transformation

LB-PEG

Glycerol	36 ml
PEG 8000	12 g
1M MgSO4	1.3 ml
LB medium	100 ml

2XTY medium

Tryptone	16 gr
Yeast Extract	10 gr
Na Cl	5 gr
H20	up to 1L

Agar plate medium

Tryptone	16 gr
Yeast Extract	10 gr
Na Cl	5 gr
Agar	8 gr
Ampicilin	1 ml
H20	up to 1 L

Solutions for Agrose gel-TBE 5X

Tris Base	27 gr
Boric Acid	13.75 gr
0.5M EDTA	10ml
H2O	up to 500 ml

2) Lysis buffer for genomic DNA preparation

	Amount 50ml	**Final conc**
Tris Hcl pH 8	2.5ml stock (1M)	50mM
EDTA pH 8	10ml stock (0.5M)	100mM
NaCl	1ml stock (5M)	100mM
SDS	10ml stock (10%)	1%

3) Western Blot solutions

Separating gel buffer stock pH to 8.8	**Amount**
Tris	90 g
SDS (0.4%)	2 g
ddH20	up to 500 ml

Ingredient	**12%**	**10%**
Lower gel buffer	3.75 ml	3.75 ml
30% Bis-acrylamide	6 ml	4.98 ml
10% SDS	150 µl	150 µl
H20	4.95 ml	6.1 ml
10% APS	67.75 µl	67.75 µl
TEMED	6.78 µl	6.78 µl
TOTAL VOL	15ml	15ml

250ml Stacking gel buffer stock pH 6.8

Tris	15.15 g
SDS (0.4%)	1gr
H2O	up to 250 ml

Ingredient	**12%acryl**	**10%**
Upper gel buffer	2.5 ml	2.5ml
Bis-acrylamide(30%)	1.5 ml	1.2ml
10% SDS	100 µl	100 µl
H20	6.8 ml	6.2 ml
10% APS	30 µl	30 µl
TEMED	10 µl	10 ul
TOTAL VOL	**10ml**	**10ml**

SAMPLE BUFFER pH 6.8

Ingredient	**Amount (3x)**	**Final Conc**
10% SDS	20 ml	4%
Glycerol	5 ml	10%
0.5M Tris	12.5 ml	0.125M
Bromophenol blue	0.01g	0.02%
dH20	50ml	

10X RUNNING BUFFER STOCK pH 8.3

Ingredient	Amount	Final Conc
Tris	30.225	250mM
glycine	142.5	1.9M
SDS	10gr	1%
H20	Up to 1L	

10x TRANSFER BUFFER STOCK pH 8.3

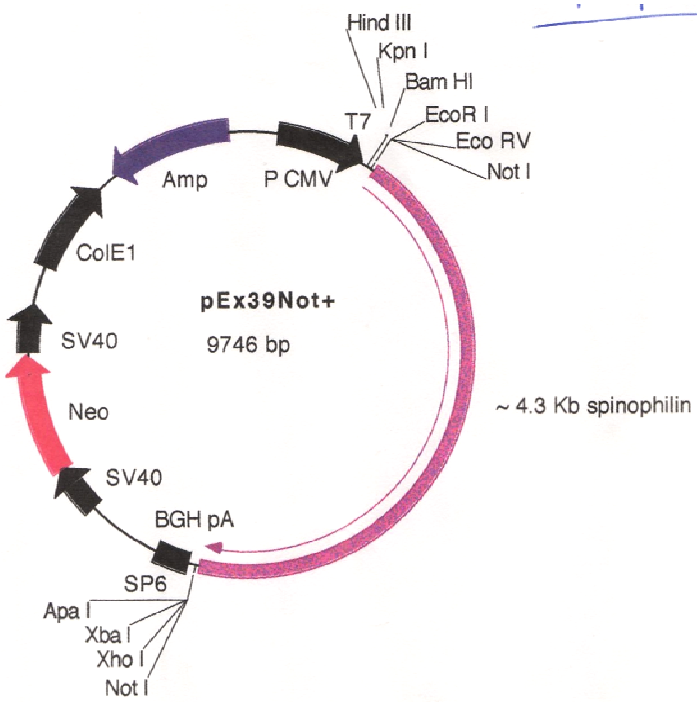
Ingredient	Amount	Final Conc
Tris	30.225	250mM
glycine	142.62	1.5M
SDS	1gr	0.1%
H20	Up to 1L	

1X TRANSFER BUFFER

Ingredient	Amount
Methanol	200ml
10x transfer buffer stock	100ml
H20 (final volume)	1L

Appendix B

Plasmid Maps- Spinophilin



Date: 8/15/96

Insert: Not I fragment from pKS39FL

Parent Vector: pCDNA3

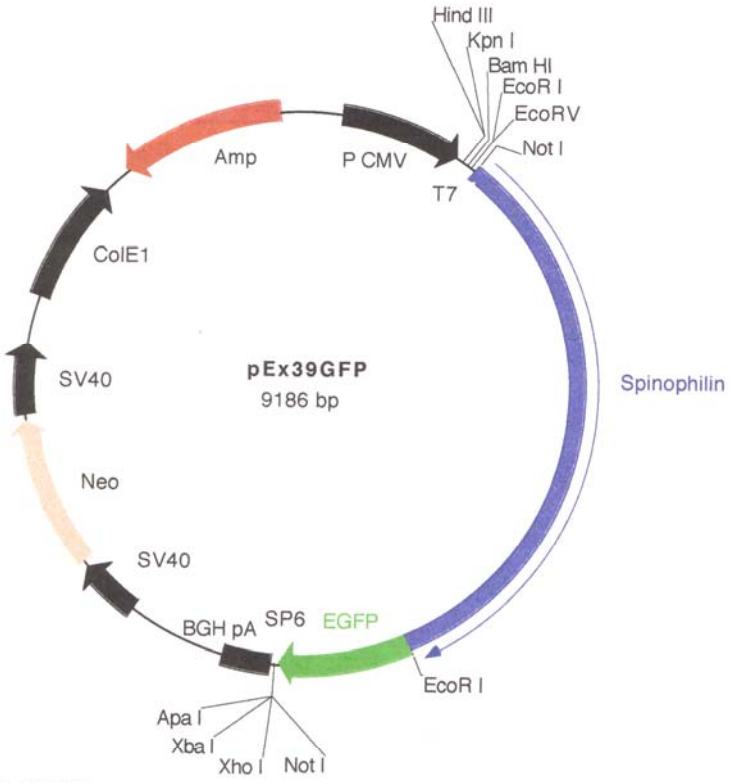
Plasmid Box: 3 B2

Frozen Stock: 4 D7

Selection: Ampicillin/G418

Notes: Sense orientation: makes full length spinophilin.

GFP-tagged Spinophilin plasmid



Date: 2/14/98

Insert: ~3.7 Kb Not I fragment from p39C'GFP

Parent Vector: pcDNA3 Not I

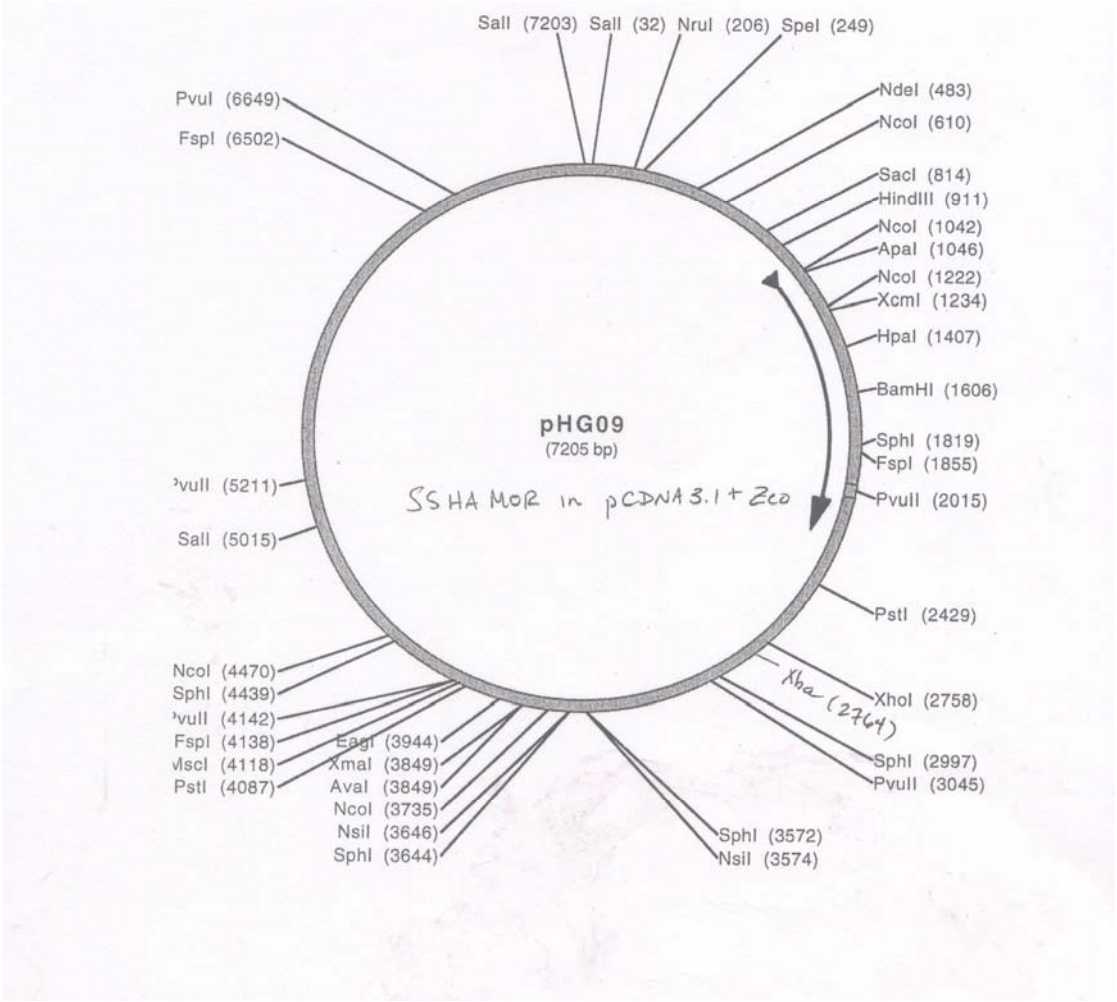
Plasmid Box: 3J10

Frozen Stock: 5F4

Selection: Ampicillin

Notes: Expresses in 293 T cells - decorates stress fibers.

HA-tagged Mu opiate Receptor (MOR) plasmid



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